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Environmental filtering decreases with fish development for the assembly of gut microbiota

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Summary

Gut microbiota typically occupy habitats with definable limits/borders that are comparable to oceanic islands. The gut therefore can be regarded as an 'island' for the assembly of microbial communities within the 'sea' of surrounding environments. This study aims to reveal the ecological mechanisms that govern microbiota in the fish gut 'island' ecosystem. Taxonomic compositions, phylogenetic diversity, and community turnover across host development were analyzed via the high-throughput sequencing of 16S rRNA gene amplicons. The results indicate that the Shannon diversity of gut microbiota in the three examined freshwater fish species all significantly decreased with host development, and the dominant bacterial taxa also changed significantly during host development. Null model and phylogenetic-based mean nearest taxon distance (MNTD) analyses suggest that host gut environmental filtering led to the assembly of microbial communities in the fish gut 'island'. However, the phylogenetic clustering of local communities and deterministic processes that governed community turnover became less distinct as the fish developed. The observed mechanisms that shaped fish gut microbiota seemed to be mainly shaped by the gut environment and by some other selective changes accompanying the host development process. These findings greatly enhance our understanding of stage-specific community assembly patterns in the fish gut ecosystem.

Introduction

For colonized gut microbiota, vertebrate digestive systems show some similarities to isolated oceanic islands (DeLong, 2014), as microorganisms in the gut ecosystem occupy a habitat with definable limits/borders that is comparable to a biogeographic island. Over the past decade, numerous studies have documented high levels of microbial diversity in vertebrate gut ecosystems (e.g., O'Hara and Shanahan, 2006; Bäckhed *et al.*, 2007; Qin *et al.*, 2010), which is especially critical for host nutrition, immunity, health, disease prevention, development, etc. (Nicholson *et al.*, 2005; Bäckhed, 2011; Velagapudi *et al.*, 2010). However, relatively little is known about how ecological processes govern the microbial community in the gut ecosystem (Dethlefsen *et al.*, 2006). Recent studies targeting gut microbiota in a model organism of zebrafish have revealed that both deterministic and stochastic processes were critical in governing microbial communities (Yan

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et al., 2012); however, the relative contributions of different processes were inconsistent and changeable throughout zebrafish development (Wong *et al*., 2015; Burns *et al*., 2016; Stephens *et al*., 2016). To the best of our knowledge, only a few studies, such as Ley *et al*. (2006), Yan *et al*. (2012), and DeLong (2014) have documented the animal gut as an 'island' ecosystem in addressing ecological issues pertaining to host-dependent microbes. As such, our understanding of the assembly mechanisms of gut microbiota remains relatively incomprehensive compared to that of plants and animals communities.

Interestingly, recent findings suggest that some microbial patterns can also be explained by the same ecological mechanisms found in plants and animals (e.g., Eiler *et al*., 2011; Stegen *et al*., 2012; Ferrenberg *et al*., 2013; Wang *et al*., 2013; Zhou *et al*., 2014). Of the four major processes (selection, dispersal, drift, and mutation/speciation) that govern the assembly of ecological communities (Vellend, 2010; Hanson *et al*., 2012), selection is a deterministic process and drift is a stochastic process, while speciation and dispersal may contribute to both deterministic and stochastic processes (Zhou *et al*., 2014). When a community is assembled under the control of stochastic processes alone, the presence of species in a community occur independent of respective niches, and site-to-site variations are unpredictable (Hubbell, 2001; Purves and Turnbull, 2010; Rosindell *et al*., 2012). However, if deterministic processes are more essential for community assembly, similar environments are expected to harbor similar communities (Diamond, 1975; Cracraft, 1988; Gotelli and McCabe, 2002). Although our knowledge of the assembly of microbial communities has increased substantially in recent years, debates on how ecological processes govern microbial communities in different ecosystems continue. For example, while Zhou *et al*. (2013) found that microbial communities in bioreactors were mainly shaped by stochastic processes, Wang *et al*. (2013) suggested that different types of aquatic bacterial communities were dominated by deterministic processes with strong habitat associations. This implies that the relative contributions of deterministic and stochastic processes to the assembly of microbial communities should vary across ecosystems. The relative importance of these processes also can shift significantly within a specific ecosystem, especially when a serious ecological disturbance occurs (Ferrenberg *et al*., 2013; Zhou *et al*., 2014). The community assembly of gut microbiota may be more complicated than that of free-living microbes, as gut microbiota are not only affected by the environment but also significantly affected by host ecology and physiology (Benson *et al*., 2010; Wong and Rawls, 2012; Bolnick *et al*., 2014).

The fish gut ecosystem presents unique features that make it especially attractive for addressing questions regarding the assembly of microbial communities. First, most fish species are oviparous and are initially separated from the outside by a chorion, and the intestine only opening to the outside several days after hatching, thus resulting in exposure to microbial colonists. Therefore, fish are theoretically microbe-free at birth, and all postnatally acquired gut microbes should migrate from surrounding environments. This differs from the process for a viviparous animal, which can obtain rich doses of its mother's bacteria during vaginal delivery and suckling, and matrilineal transmission has been shown to have significant effects on the postnatal acquisition of microbiomes (Penders *et al*., 2006; Neu and Rushing, 2011). Additionally, as all activities carried out by fish (e.g., feeding, defecation, and breeding) take place in water, interactions between fish and water environments may be much more direct than those between mammals and terrestrial environments (De Schryver and Vadstein, 2014). Another important reason for focusing on the fish gut ecosystem pertains to the fact that fish account for nearly half of all vertebrate diversity (Nelson, 2006), thus creating a variety of habitats (each species may represent a particular gut type) for addressing community assembly mechanisms in different habitats. Moreover, knowledge of fish gut microbiota assembly also can be extended to the mammalian gut ecosystem, as there is a considerable degree of microbial overlap between fish and mammals (Sullam *et al*., 2012), and their intestinal environments are also known to be similar in some key respects (Stephens *et al*., 2016).

The present study aims to reveal the major ecological processes that govern microbial communities in the gut of main aquaculture fish species (considering variations in fish taxa, feeding habits, diets, trophic levels and developmental stages) in China. Our previous investigations (Li *et al*., 2012; 2014) and some other studies (e.g., Rawls *et al*., 2006; Roeselers *et al*., 2011) on fish gut microbiota suggested that the host (in addition to diet [Sullam *et al*., 2015]) considerably affected community composition and turnover patterns. Therefore, gut microbiota assembly in fish may be primarily controlled by deterministic processes due to host-dependent restrictions. Individuals of the same fish species of a particular developmental stage (which may share similar gut habitat) tended to be colonized by similar gut microbiota from the same regional species pool (Fig. 1A). Additionally, the ecological processes that contribute to the assembly of gut microbiota should also be associated with fish development (Fig. 1B), which has been discussed earlier (Yan *et al*., 2012; Burns *et al*., 2016). As expected, we found that all of the three investigated fish species harbored distinct microbial communities at different developmental stages but shared similar communities during a particular stage, thus supporting the notion that determinism is much more important for the assembly and turnover of freshwater fish gut microbiota.

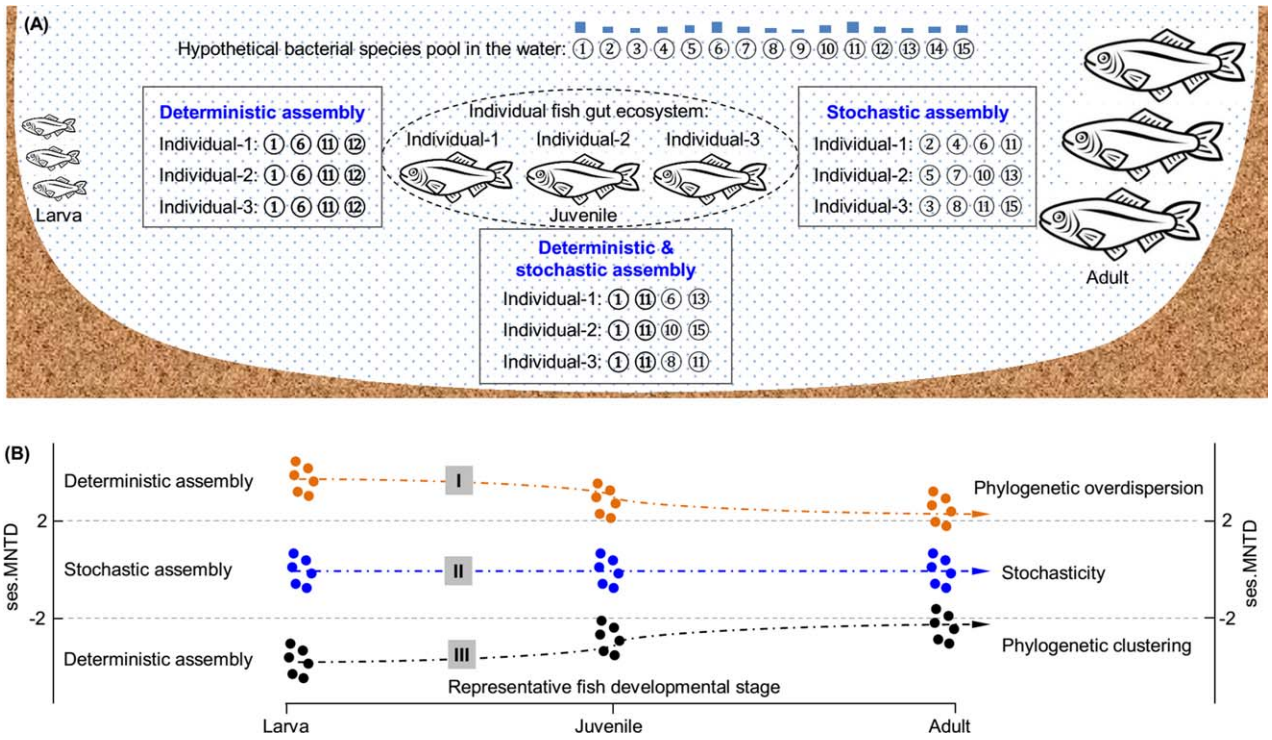

Fig. 1. Schematic representation of how ecological processes govern the assembly of a local community in the fish gut ecosystem.
A. Deterministic and stochastic assembly will result in the development of different communities in similar gut environments. Individuals of the same fish species at a particular developmental stage (which may share similar gut habitats) are expected to colonize similar gut microbiota from the same bacterial species pool when community assembly is governed by deterministic processes. By contrast, when community assembly is only determined by stochastic factors, the gut microbiota are expected to differ among individuals due to stochasticity factors. When both deterministic and stochastic processes contribute significantly to community assembly, similarities and differences in community compositions are detectible. The numbers ① to ⑮ represent a hypothetical bacterial pool of available species in water environment, and the corresponding bars shown above denote their relative abundance. Microbial species that are expected to deterministically migrate into the gut are shown in bold, and un-bolded species denote bacteria that stochastically colonized the fish gut.
B. Deterministic assembly tends to weaken with host development (lines I and III) or is randomly assembled over a lifetime (line II) as reflected by the standardized effect size of the mean nearest taxon distance (ses.MNTD). Horizontal dashed lines denote that ses.MNTD = −2 or 2; values beyond this point are considered statistically significant for deterministic processes (phylogenetic clustering or phylogenetic overdispersion), whereas those falling between them suggest that microbial taxa in the gut appeared stochastically.

Phylogenetic analyses further showed that host gut environmental filtering determined community assembly in the fish gut 'island' ecosystem but phylogenetic clustering decreased with host development.

Results

Composition and general turnover of fish gut microbiota

Through our current sequencing efforts, we detected a total of 3,559, 1,804 and 2,549 OTUs (UPARSE, 97% cutoff) from the *Ctenopharyngodon idellus, Siniperca chuatsi* and *Silurus meridionalis* samples, respectively. Proteobacteria OTUs accounted for 33.35–34.95% of the detected taxa in the three fish species examined. The beta-diversity analysis, which was based on the overall community composition or on a particular taxonomic group of gut bacteria, showed clear changes between host developmental stages (e.g., larval stage, juvenile, adult, Supporting Information Fig. S1). However, the larval stage also can present considerable changes in community composition in accordance with morphological and histological differentiations of fish digestive system (Wu *et al.*, 2007; Li *et al.*, 2013). For example, a dissimilarity test based Bray-Curtis distances suggests that the gut microbiota differed significantly (MRPP and PERMANOVA, $P < 0.05$) between any two of compared stages (Table 1). These host development-dependent community patterns were also confirmed by the DCA ordination based on the taxonomic composition or through a PCoA ordination calculated from weighted UniFrac distances (Fig. 2), revealing that the gut microbiota of fish individuals of the same stages were generally grouped together. More interestingly, we found that Shannon diversity levels significantly (regression models, $P < 0.05$) decreased as the host developed, regardless of whether the overall community composition was considered (Fig. 3A–C) or just focus on dominant taxonomic phyla such as Proteobacteria (Supporting Information Fig. S2A–C) and Firmicutes (Supporting Information Fig. S3A-B).

Table 1. Bray-Curtis distance-based dissimilarity test showing differences in gut microbiota between host developmental stages.

	MRPP		PERMANOVA	
	Delta	*P*	*F*	*P*
Ctenopharyngodon idellus				
Larva (1–4 dph) vs. Larva (5–30 dph)	0.790	**0.001**	7.319	**0.001**
Larva (1–4 dph) vs. Juvenile	0.744	**0.001**	6.374	**0.001**
Larva (1–4 dph) vs. Adult	0.692	**0.001**	12.384	**0.001**
Larva (5–30 dph) vs. Juvenile	0.741	**0.001**	6.075	**0.001**
Larva (5–30 dph) vs. Adult	0.696	**0.001**	12.654	**0.001**
Juvenile vs. Adult	0.549	**0.001**	8.981	**0.001**
Siniperca chuatsi				
Larva (1–13 dph) vs. Larva (18–23 dph)	0.645	**0.001**	13.977	**0.001**
Larva (1–13 dph) vs. Adult	0.618	**0.001**	9.149	**0.001**
Larva (18–23 dph) vs. Adult	0.707	**0.001**	6.104	**0.001**
Silurus meridionalis				
Larva (3–13 dph) vs. Larva (18–33 dph)	0.573	**0.001**	23.360	**0.001**
Larva (3–13 dph) vs. Adult	0.493	**0.001**	20.952	**0.001**
Larva (18–33 dph) vs. Adult	0.529	**0.001**	20.764	**0.001**

MRPP: multiple-response permutation procedure; PERMANOVA: permutational multivariate analysis of variance; dph: day post-hatching. *P* values < 0.05 in bold.

The relative abundance of the detected bacteria also suggests that Proteobacteria were the most abundant phylum in all three of the investigated fish species throughout their lifetime. In brief, the first larval stage of herbivorous *C. idellus* (1–4 dph) and carnivorous *S. chuatsi* (1–13 dph) harbored similar but very high degrees of Proteobacteria abundance (above 50%) that were significantly higher than those of carnivorous *S. meridionalis* from 3 to 13 dph (approximately 30%, Supporting Information Fig. S4). However, the relative abundance of Proteobacteria in the two carnivorous species increased further during the adult stage (Supporting Information Fig. S4). Moreover, the two carnivorous species initially were also dominated by the Firmicutes, but abundance levels decreased significantly (ANOVA, $P < 0.05$) after 18 dph, when Cyanobacteria or Fusobacteria significantly (ANOVA, $P < 0.05$) increased (Supporting Information Fig. S4B-C). However, Fusobacteria in the herbivorous *C. idellus* samples were relatively higher in abundance during the juvenile stage. Additionally, Firmicutes in the herbivorous *C. idellus* samples were initially less dominant from the hatching stage to the juvenile stage and only increased significantly (ANOVA, $P < 0.05$) at the adult stage (Supporting Information Fig. S4A).

Core gut microbiota levels varied across fish developmental stages and differed across hosts feeding habits

The composition of gut microbiota at phylum level showed clear patterns with fish development, this section we go ahead to present the variation at a more refined taxonomic level (i.e., genus or OTU). The taxa shared by most ($\geq$90%) of gut communities with relatively high abundance were generally treated as core gut microbiota (Qin *et al*., 2010; Roeselers *et al*., 2011), here we focused on the shared taxa with relative abundance above 1% to further examine core bacterial shifts across host development stages. First, we found clear core bacterial turnover patterns across fish development stages as visualized by the heat maps, which show that almost all of the individual samples were clustered into groups according to each host's respective developmental stage due to clear bacterial patterns (Fig. 4). Generally, similar dominant genera assembled in the fish individuals during a particular stage and thus generated similar color (denoting relative abundance) patterns on the heat map, but patterns across stages varied. For example, the co-varying taxa of *Lactococcus, Leucanostoc, Weisella* and *Acinetobacter* were found together in relatively large quantities in the youngest larval cohort of the two carnivorous species (i.e., *S. chuatsi* and *S. meridionalis*) but were relatively absent in the older individuals. These dominant genera, which in total accounted for 47.4–89.3% of the bacterial abundance, showed clear variations across host developmental stages (Fig. 5, Supporting Information Table S1A). Moreover, we found a broader array of dominant taxa in the herbivorous *C. idellus* than in the carnivorous *S. chuatsi* and *S. meridionalis*. The most dominant genera (e.g., highlighted in dark red in Supporting Information Table S1) always varied significantly (ANOVA, $P < 0.05$) between host developmental stages. Moreover, the compositions of these dominant genera in the adult individuals were relatively simpler but were more focused (the most highly abundant genus accounted for 37–69% of the abundance: e.g., *Aeromonas* in *C. idellus*, *Serratia* in *S. chuatsi*, *Escherichia* in *S. meridionalis*) than those found in the larvae (Figs. 4, 5 and Supporting Information Table S1A).

To determine how diet features affected fish gut microbiota in the adult individuals, seven fish species representing herbivores, omnivores, and carnivores were compared. Interestingly, we found that the Shannon diversity of gut microbiota from fish at the highest trophic level (carnivores) was significantly ($P < 0.05$) lower than that found for the herbivorous and omnivorous fish. The abundance of the dominant Proteobacteria tended to increase with fish trophic levels (from herbivores to carnivores), while Firmicutes abundance generally decreased with trophic levels (Supporting Information Fig. S5). The two most common phyla (i.e., Proteobacteria and Firmicutes) accounted for 74.1–98.2% of the total observed abundance, and they differed significantly (ANOVA, $P < 0.05$) between the herbivorous and carnivorous fish. Bacteroidetes were relatively frequent in the adult herbivores but were nearly absent in the adult omnivorous and

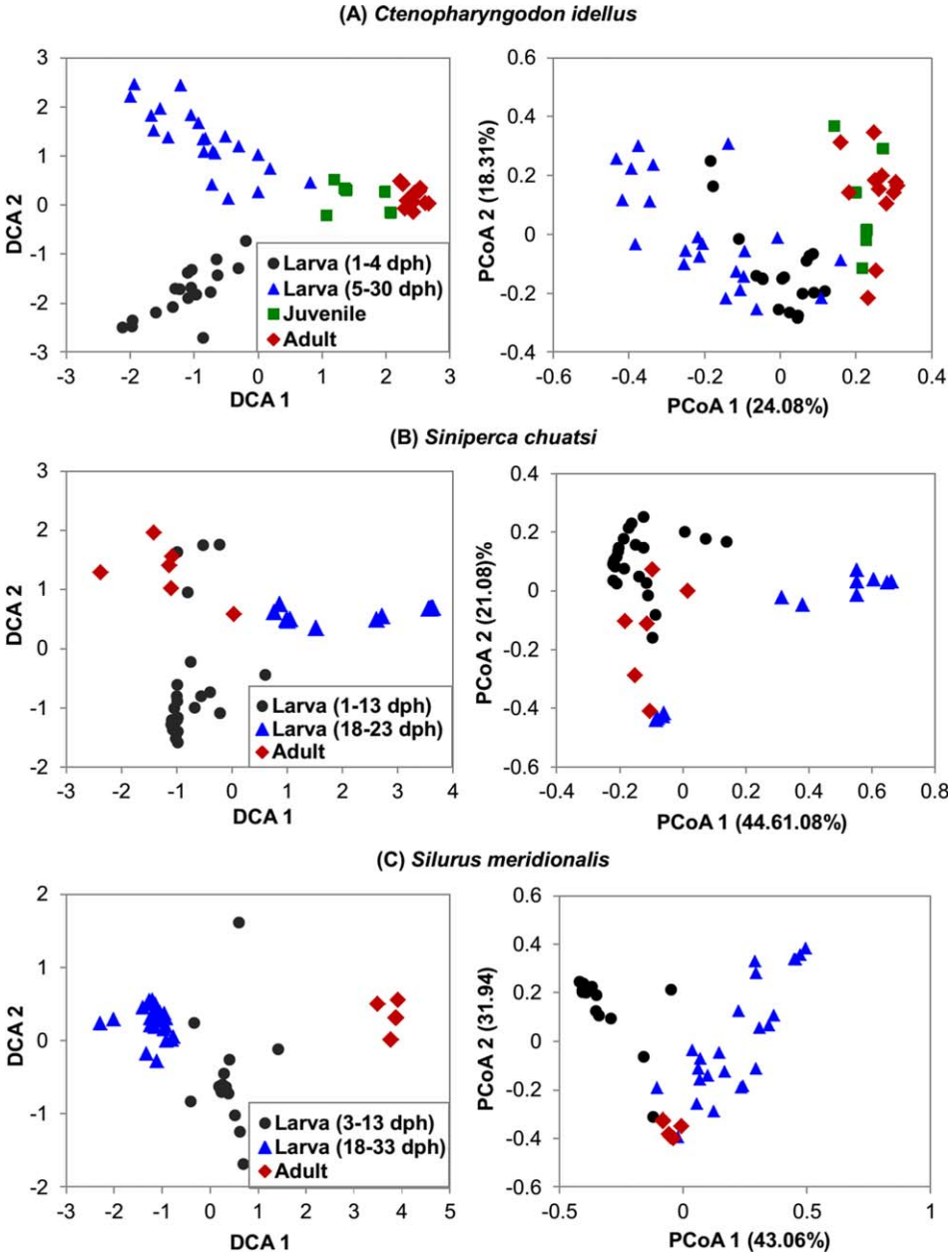

Fig. 2. Detrended correspondence analysis (DCA) and principal coordinate analysis (PCoA) illustrating community dissimilarities throughout host development based on the taxonomic composition (left) and weighted UniFrac distances (right) of gut microbiotas.

carnivorous fish (Supporting Information Fig. S5). We found that 18 genera were core and abundant bacteria (> 1% of total abundance), accounting for 54.4–85.2% of the total bacterial abundance (Fig. 5D).

Ecological processes governing the assembly of fish gut microbiota

To test the possible effects of surrounding water on initial gut microbiota colonization patterns, fertilized *C. idellus* eggs of the same parentage were randomly distributed into four different environments (for further details, see Supporting Information Table S2) and then hatched *in situ*. Unexpectedly, we found that community patterns of bacteria colonized in the fish gut did not significantly correlate with the water bacterial communities and were only developmental stage-dependent. The Bray-Curtis distance-based PERMDISP results also significantly ($P < 0.05$) vary from the null random expectation for each stage (Table 2). Phylogenetic signals results (Supporting Information Fig. S6) suggested that it would be better to focus on the closely related bacterial species/OTUs in further phylogenetic analysis. As that, we found the composition of closely related gut microbiota was mainly governed by the environmental filtering, as most ses.MNTD values for the local communities were less than −2. However, this

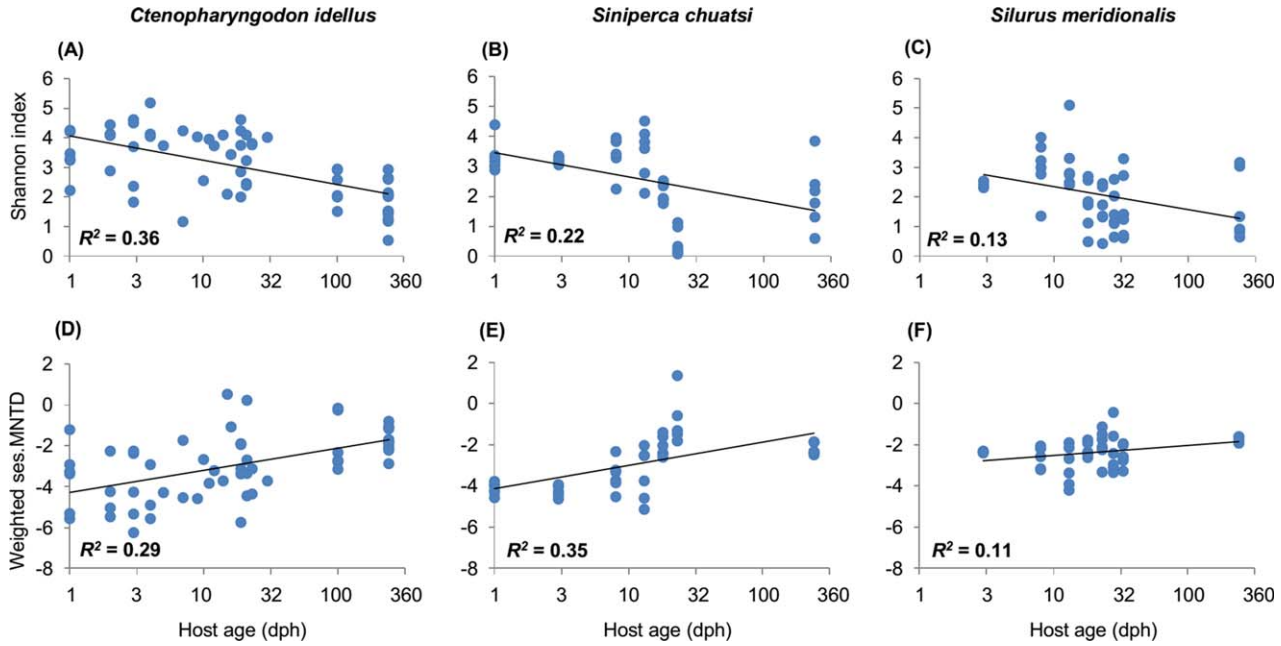

Fig. 3. The alpha-diversity (Shannon index, A–C) and environmental filtering (host selection) of gut microbiota (as determined by the weighted standardized effect size of the mean nearest taxon distance (ses.MNTD), D-F) significantly decreased with host development (all P values < 0.05).

deterministic process that determined community assembly patterns tended to weaken significantly (regression models, $P < 0.05$) with host development (Fig. 3D–F). This was also found to be true at a particular taxonomic level after separately analyzing the two most dominant phyla (Proteobacteria and Firmicutes) (Supporting Information Figs. S2D–F and S3D–F).

A plot of pairwise βMNTD values versus fish development results also shows that community dissimilarities significantly (regression models, $P < 0.05$) increased with increasing host developmental day-intervals (Supporting Information Fig. S7). In other words, communities tended to differ more among those individuals with longer intervals than between those of the same stage or with shorter intervals. Taken together, these data suggest that ecological processes that determine the community turnover of gut microbiota are also dependent on fish development. By further quantifying the relative contributions of major ecological processes that structure gut microbiota, we found that processes regulating community turnover differ considerably between larvae and adults (Fig. 6). Generally, drift process is much more pronounced in adults, while selection and dispersal play a much more important role in the assembly of gut microbiota in larvae. Moreover, at the larval stage (the first month of post hatching), different fish species showed different patterns. For example, dispersal limitation and homogeneous selection processes were the two main processes that governed microbial community turnover patterns in the larvae of *C. idellus* (totally 94%) and *S. chuatsi* (totally 77%), but homogeneous selection (60%) plays a much more significant role in larval *S. meridionalis* (Fig. 6). However, drift and homogeneous selection were consistently important for governing the community patterns of gut microbiota in the adult individuals, and no clear variations were found between host feeding habits or among different fish species (Fig. 6; Supporting Information Fig. S8).

Discussion

Understanding the mechanisms that underlie community assembly is regarded as centrally important in the field of community ecology. However, most previous studies have focused on plants and animals, and the processes that drive bacterial community assembly remain poorly understood (Burke *et al.*, 2011). Recently, knowledge of plant and animal community assembly has also been extended to microorganisms with the help of metagenome-based techniques (e.g., Stegen *et al.*, 2012; 2013; Wang *et al.*, 2013; Zhou *et al.*, 2013; 2014). However, how environmental microbes colonize the fish gut ecosystem and which ecological processes drive this assembly remains unknown. Most culture-independent studies on fish gut microbiota have focused on examining which environmental factors (e.g., diets, habitats, hosts) mostly affect microbial communities and how these microbes interact with their hosts (e.g., Roeselers *et al.*, 2011; Sullam *et al.*, 2012; Bolnick *et al.*, 2014). Moreover, most of these studies only analyze a particular host species during a single developmental stage. However, different host species

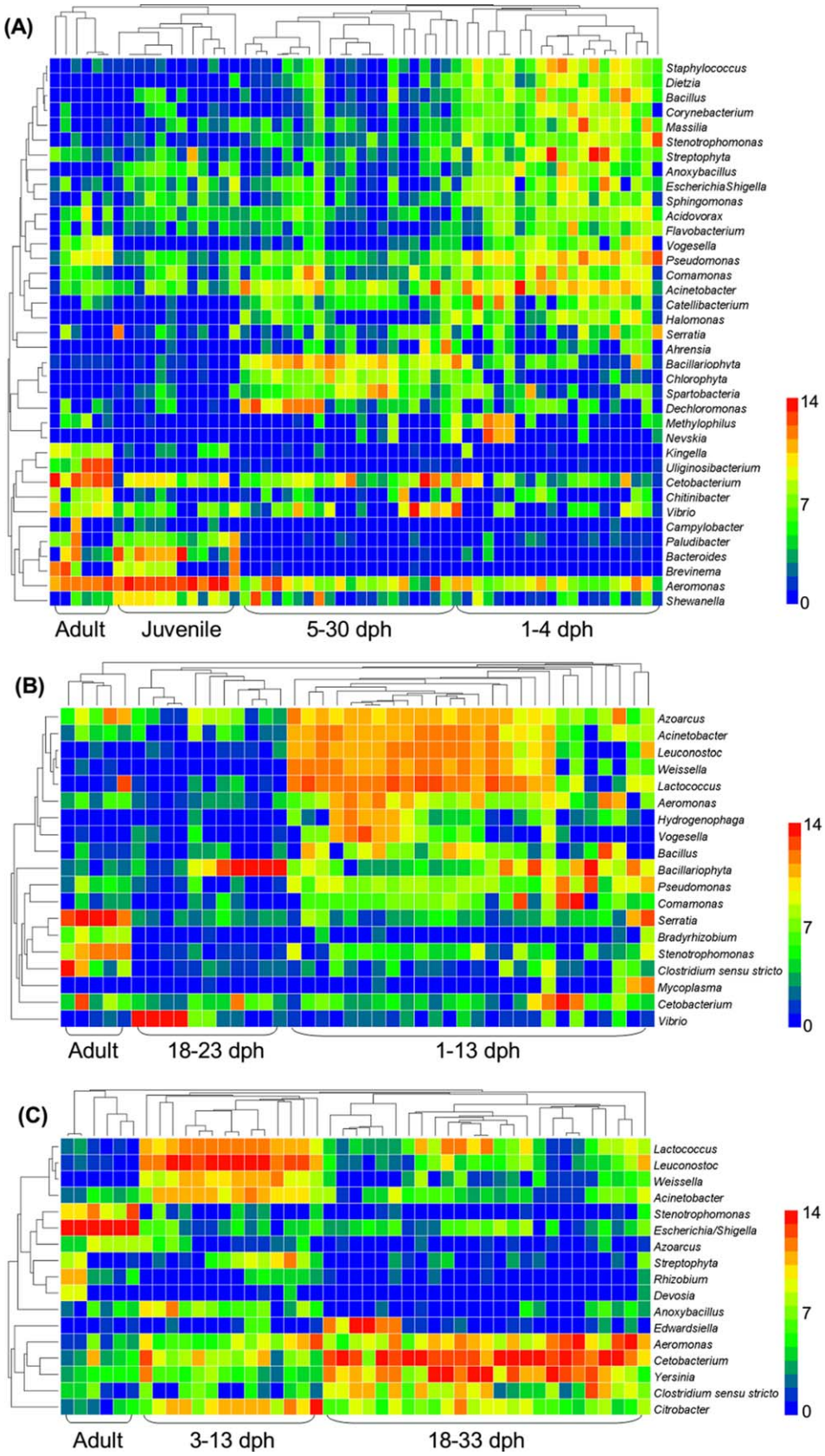

Fig. 4. Heat map (complete-linkage clustering) showing the dominant genera of gut microbiota in each *Ctenopharyngodon idellus* (A), *Siniperca chuatsi* (B) and *Silurus meridionalis* (C) individual.

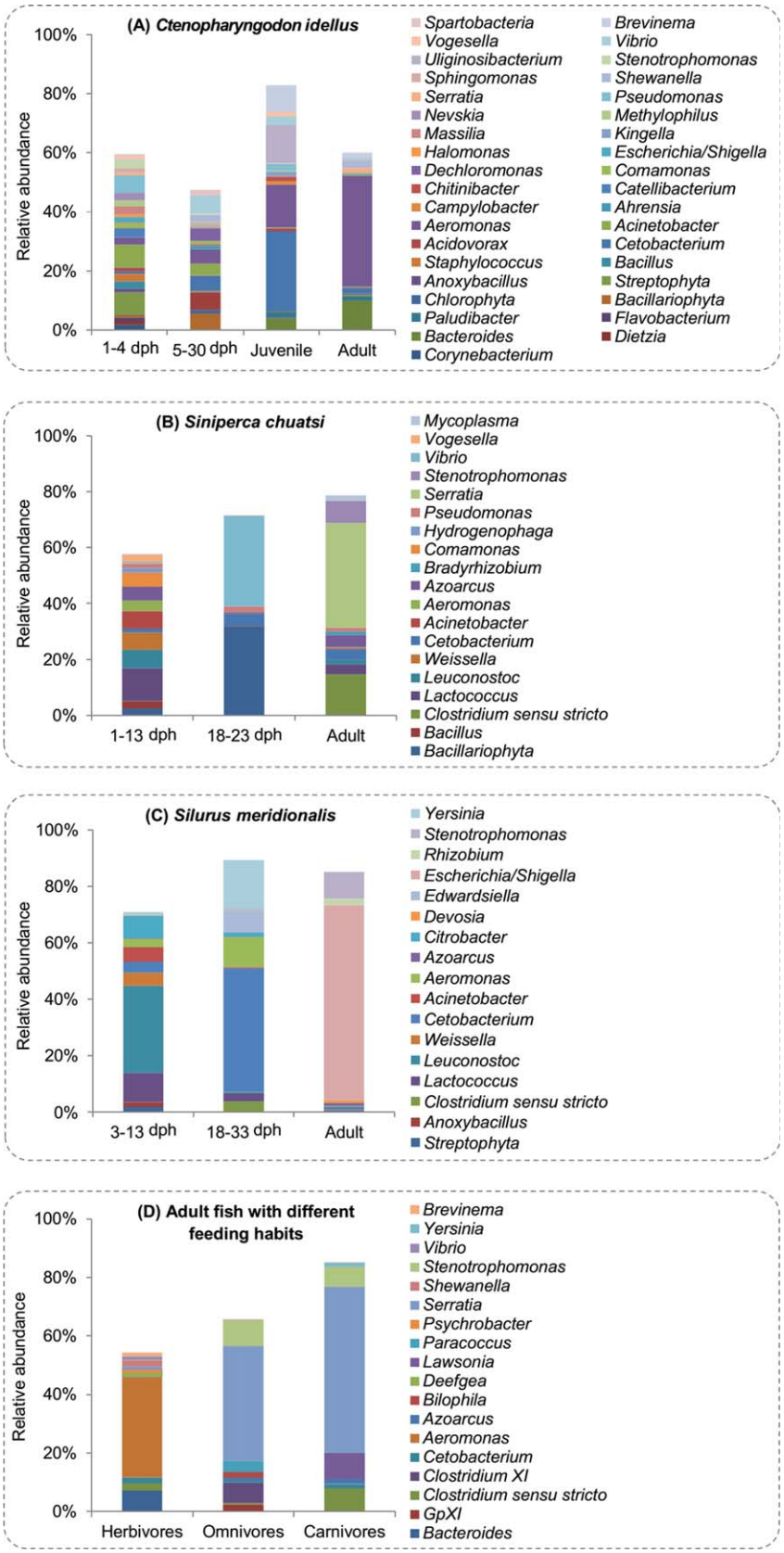

Fig. 5. Relative abundance of the dominant microbial genera detected in the guts of fish at different developmental stages (A–C) or with different feeding habits (D). Mean values of samples collected from each stage (A–C) or for different feeding habits (D) are plotted. For ANOVA statistics that show differences between the pairwise comparisons, please refer to Tables S1 in the Supporting Information section.

Table 2. Bray-Curtis distance-based significance test of centroid differences between the observed communities and the null model simulations for each stage

	Actual centroid	Null centroid	*F*	*P*
Ctenopharyngodon idellus				
Larva (1–4 dph)	0.548	0.605	8.839	**0.005**
Larva (5–30 dph)	0.544	0.624	22.667	**0.000**
Juvenile	0.366	0.612	7.352	**0.022**
Adult	0.352	0.654	32.445	**<0.001**
Siniperca chuatsi				
Larva (1–13 dph)	0.407	0.609	20.337	**<0.001**
Larva (18–23 dph)	0.536	0.663	8.883	**0.007**
Adult	0.430	0.592	7.457	**0.021**
Silurus meridionalis				
Larva (3–13 dph)	0.370	0.645	25.871	**< 0.001**
Larva (18–33 dph)	0.405	0.659	75.206	**< 0.001**
Adult	0.219	0.574	99.457	**< 0.001**

A permutational analysis of multivariate dispersions (PERMDISP) was conducted; dph: day post-hatching. *P* values < 0.05 in bold.

showed clear differences even they were from the same environment (Li *et al*., 2012; 2014), host physiological development also has a significant effect on gut microbiota independent of other factors (Stephens *et al*., 2016).

Our previous study on four fish species occupying the larval stage (7 dph) that were hatched and reared in the same water environment (i.e., with an identical microbial species pool) showed that hosts constitute the main determinant of intestinal microbiota (Li *et al*., 2012). In analyzing eight different fish species occupying the adult stage that were collected from the same lake, we also found that hosts significantly affect the composition of gut microbiota (Li *et al*., 2014). Although other factors such as diets, living environments, and selective pressures from gut habitats may also affect fish gut microbiota (Rawls *et al*., 2006; Li *et al*., 2012; Yan *et al*., 2012; Wong and Rawls, 2012; Ni *et al*., 2014; Sullam *et al*., 2015; Wong *et al*., 2015), fish reared under considerably different conditions can selectively assemble some core microbes that shared by most gut communities and with relatively high abundance (e.g., Roeselers *et al*., 2011; Wu *et al*., 2012). More recently, studies of zebrafish have offered an initial view into lifelong gut microbiota assembly processes over the course of fish development (Wong *et al*., 2015; Burns *et al*., 2016; Stephens *et al*., 2016). The present study directly focused on gut microbiota assembly and turnover mechanisms in different types of fish over the course of host development.

Because microbes that first colonize the gut have long-term effects on the host (Costello *et al*., 2012; De Schryver and Vadstein, 2014), it is especially important to understand which microbes are the first 'winners' that migrate into a completely new gut ecosystem (the gut is initially microbe-free before it is opened to the surrounding environment) and then function as gut residents. We found that gut microbiota patterns are not significantly correlated with the environmental community patterns (Supporting Information Table S2), but they only exhibit significant host stage-dependent patterns (Table 1). Our findings are consistent with those observed in other fish such as *Poecilia sphenops* (Schmidt *et al*., 2015). Sullam *et al*. (2012) also suggested that rearing environments have no significant effects on the compositions of fish gut microbiota. Similar results were also found for gut microbiota assembly processes in mammals, where exogenous selection always generates more Firmicutes regardless of the original inputs involved (Seedorf *et al*., 2014). Of all of the factors that may contribute to gut microbiota variations, host development processes have been acknowledged as one of the most important factors for both mammals and fish (Avershina *et al*., 2016; Wong *et al*., 2015). Taken together, fish may selectively filter particular microbial members from the exogenous species pool (as illustrated in Fig. 1A) to function as gut residents at different developmental stages. In other words, communities are deterministically assembled rather than randomly adopting microbial hitchhikers from ingested food or water environments.

Although the feeding for herbivorous *C. idellus* only changed after 5 dph (Supporting Information Fig. S1), we found that gut microbiota showed a significant shift before the food changing (i.e., 4 dph) and then remained relatively stable from 5 to 30 dph, suggesting host development rather than feeding drives the initial colonization of gut microbiota. This rapid change after 4 dph also make sense, as symbiotic bacterial populations in the fish gut can multiply from tens to tens of thousands of cells in only a few hours (Jemielita *et al*., 2014). By contrast, the first significant community turnover event for the carnivorous *S. chuatsi* and *S. meridionalis* individuals occurred relatively later (after 13 dph) (Table 1, Fig. 2). This data is consistent with morphological and histological differentiations of the stomachs and intestines of these carnivorous fish (Wu *et al*., 2007; Li *et al*., 2013), suggesting that fish gut microbiota is really host developmental stage-dependent. Stephens *et al*. (2016) also supported the notion that morphological changes occurring during zebrafish development are dominant drivers of gut microbiota changes. The abundance weighted null model test (PERMDISP) results further confirm that gut microbiota assemblages of each stage differ significantly from the null random expectation (Table 2). These data suggest that composition of fish gut microbiota mainly assemble deterministically rather than stochastically. Our finding is consistent with the results presented by Schmidt *et al*. (2015) and Stephens *et al*. (2016), who also found that deterministic processes appear to drive fish microbiome assembly. Of course, some studies have also found substantial individual variations in the intestinal microbiota of larval (e.g., Fjellherim *et al*., 2012) or adult fish (e.g., Burns *et al*., 2016), suggesting that stochastic process contributions should also be

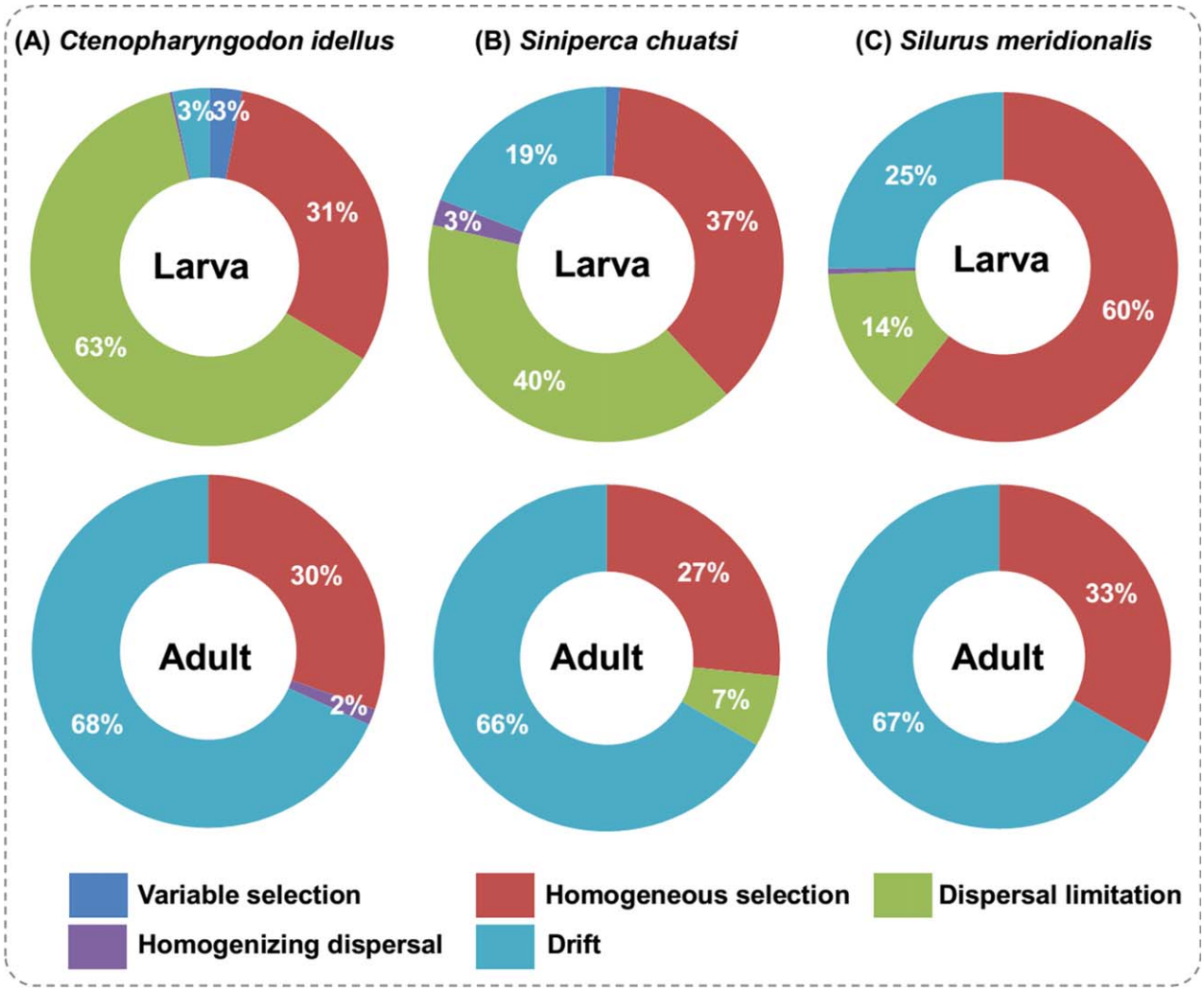

Fig. 6. Summary of the contribution of the ecological processes that determine community assembly of gut microbiota in larval and adult fish.

considered. Therefore, it is especially important to further quantify the relative importance of each ecological process in governing the assembly and turnover of microbial communities throughout host development.

Fortunately, a relatively new ecological framework based on phylogenetic distance has been developed to determine and quantify processes that govern microbial communities (Stegen *et al*., 2012; 2013; 2015). Although some methodological artifacts (e.g., PCR-bias, sample size, and DNA sequencing errors) may affect the processes estimated using this framework (Stegen *et al*., 2015), it have been acknowledged as effective means of analyzing the assembly of human gut microbiota, soil, aquatic or some other microbial communities (e.g., Martínez *et al*., 2015; Dini-Andreote *et al*., 2015). This study is the first to use this newly developed framework to address the assembly mechanisms of fish gut microbiota. Phylogenetic signals (Mantel correlation, $P < 0.05$) of all the three examined fish species were only significant at a relatively short distance (Supporting Information Fig. S6), suggesting that the positive relationships between bacterial ecological differences and phylogenetic distances should only occur among close relatives (Stegen *et al*., 2012). Wong *et al*. (2015) also found that assemblages in zebrafish gut microbiota are often shared among closely related taxa. By analyzing the phylogenetic composition of close relatives, we found environmental filtering dominates assemblages of microbial communities in the fish gut ecosystem at local scales as indicated by negative ses.MNTD values with low quantiles ($P < 0.05$) (Webb *et al*., 2002; Wang *et al*., 2013). However, the phylogenetic clustering tends to weaken with fish development as indicated by the significant decrease of absolute magnitude of ses.MNTD (larger value of |ses.MNTD| reflects greater effects, Wang *et al*., 2013).

Several factors may contribute to this kind of community assembly patterns in fish gut ecosystem. First, newly

formed gut systems (short time after hatching) of the same fish species may be similar across individuals due to population genetics, thus resulting in the filtering of similar gut microbiota from the same environmental species pool as a result of deterministic processes (as illustrated in Fig. 1A). In turn, these selected bacteria can help the host digest food or resist disease as its digestive and immune systems are just under development. However, this does not mean that initial 'winners' (i.e., colonizers) selected by the host can maintain their advantage over the course of the host's life. When gut resources and niche availability levels change during the host development stage, dominant microbial groups in the gut also change (Bolnick *et al*., 2014). Gut habitats may change considerably throughout host development and may thus favor different microorganisms (DeLong, 2014). Our findings are also consistent with the stage-specific zebrafish intestinal microbiota (Stephens *et al*., 2016). Additionally, larval individuals generally live in relatively small water areas (sharing a high similarity of regional species pool) with relatively high density, and their direct defecation into the water all can encourage the exchange of gut microbes (especially beneficial members) among individuals (Johnson and Winquist, 2011). Beneficial bacterial cells then can quickly grow a large population (Jemielita *et al*., 2014), causing different larval individuals of a particular stage to share similar dominant gut microbiota.

The βMNTD results show that the beta-diversity of gut microbiota increased throughout the host developmental (Supporting Information Fig. S7) in all three investigated fish species, suggesting that gut microbiota variation levels increase with fish development. These data are in agreement with previous works on zebrafish intestinal microbiota, which have been shown to assemble into distinct communities throughout host development and which become increasingly differentiated (Stephens *et al*., 2016). We found that the relative importance of ecological processes differs between larvae and adults. While stochasticity (drift) levels significantly increased in the adults, determinism (selection) levels in the larvae (especially in the carnivorous *S. chuatsi* and *S. meridionalis*) were stronger than those found in the adults (Fig. 6). The development of the fish digestive and immune systems, which may significantly affect gut microbiota (Benson *et al*., 2010; Costello *et al*., 2012), may significantly contribute to these ecological patterns. For example, various digestive enzymes are available in the guts of adults, and these enzymes may considerably help fish digest food (Alarcón *et al*., 1997; Portella and Dabrowski, 2008), whereas larvae, which have incomplete digestive systems, may be more dependent on microbes for digesting food. Moreover, the larvae feed on various planktonic organisms during early stages, and diverse diets may also contribute somewhat to the observed higher alpha-diversity levels in larvae. The adults selectively feed on simpler and relatively stable food, and therefore, may only need to ingest particular microorganisms to digest food that is indigestible by their own enzymes. Thus, high levels of alpha-diversity observed at the larval stage may be due to the selection of different types of microbes with various desired functional traits from the environment (Fig. 3A–C). This is consistent with processes found in zebrafish gut ecosystems, where bacterial richness also decreases with host age (Wong *et al*., 2015; Stephens *et al*., 2016). These findings collectively suggest that the mechanisms that control fish gut microbiota assembly are largely dependent on host development. Further studies will be needed to determine how ecological processes impose the selection of microbiota in fish gut ecosystems and how gut microbiota service the host. This information will be especially critical for the further microbial management of more beneficial gut microbiota than those that are naturally assembled, thus further assisting hosts by preventing exposure to pathogenic diseases and/or by providing access to dietary nutrients.

In conclusion, the fish gut microbiota do not simply reflect surrounding environments or host diets but appear to have complex relationships with host ontogenetic differences. We found that bacterial community assembly in the fish gut 'island' ecosystems is mainly governed by the deterministic process of environmental filtering. However, the phylogenetic clustering of local communities tends to decrease with host development, and community turnover levels in larvae and adults are also dominated by different ecological processes due to gut environment changes and other selective variations in host development. This study greatly expands our understanding of ecological processes that govern microbial community assembly in the fish gut 'island' ecosystem over the course of host development. Moreover, as fish gut microbiota present numerous similarities with those of mammals (Sullam *et al*., 2012) and as their intestinal environments are also similar in some key respects (Stephens *et al*., 2016), the findings of the present study also can contribute further insights to community assembly studies of mammalian gut ecosystems.

Experimental procedures

Experimental animals and fish sampling

To study the assembly mechanisms of microbial communities in the fish gut ecosystem, three major aquaculture fish species in China (i.e., herbivorous *Ctenopharyngodon idellus*, carnivorous *Siniperca chuatsi*, and *Silurus meridionalis*) were examined from the larval stage (1–33 day post-hatching, dph) to the adult stage (360 dph). For the larval individuals, samples were taken at different time points for the three fish species as indicated in Supporting Information Fig. S1. Adult individuals of four additional fish species (i.e., herbivorous *Carnis megalobramae*, omnivorous *Carassius auratus* and *Cyprinus carpio*, and carnivorous *Canna micropeltes*), which were collected from the same water environment (Poyang

Lake) as that of the adult *C. idellus*, *S. chuatsi*, and *S. meridionalis* samples, were analyzed to determine whether feeding habits affected community assembly patterns in the fish gut ecosystems. All of the adult individuals used in this study were collected in the summer of 2012.

Larval *C. idellus* were hatched and reared *in situ* by setting net cages (1 mm mesh net) in different natural environments (i.e., Niushan Lake, Wuhu Lake, pond in Niushan Lake Experimental Station, and pond in Guanqiao Experimental Station) to address possible environmental effects on the assembly of gut microbiota. In each net cage, only individuals at the same developmental stage were raised, and sampling for this species began at 1 dph and then occurred daily for the first 5 days. Following that, samples were taken every 4 days till 21 dph, and the last sampling at larval stage was taken at 30 dph. For the first five days, larvae were fed boiled egg yolk three times a day (6:00, 14:00, and 22:00) and were then fed commercial fish powder. An additional juvenile stage samples were also collected before the adult stage (Supporting Information Fig. S1). Water samples at the larval stage were sampled for the analysis of environmental community compositions. Approximately 10 liters of water was collected using a polypropylene bucket, and one liter was sequentially filtered through 1.2 μm (Whatman, NJ, USA) and 0.22 μm filters (Millipore, MA, USA) for the collection of microbial cells as described previously (Yan *et al.*, 2015). The filters were then stored at −20°C until DNA extraction.

The hatched *S. chuatsi* and *S. meridionalis* were reared in ponds ($10.0 \times 3.5 \times 1.0$ m^3) at the Hubei Fishery Science Institute and at Wuhan Bihai Farm, respectively. The animals of each species at the same developmental stage were raised in a pond for our experiment. For the first three days, larvae were fed boiled egg yolk three times a day (6:00, 14:00 and 22:00) and were then fed baby dace (*S. chuatsi*) and water earthworms (*S. meridionalis*). Sampling began at 1 dph and 3 dph for *S. chuatsi* and *S. meridionalis*, respectively, and then sampling was conducted every 5 days (Supporting Information Fig. S1). Intestine sampling (described below) was performed immediately following fish collection, and the collected gut samples were stored at −20°C until DNA extraction. All protocols involved in the animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Hydrobiology of the Chinese Academy of Sciences (Approval ID: Keshuizhuan 08529).

Gut sample collection and DNA extraction

The intestines and/or stomachs of the small larval individuals were removed aseptically under a dissecting microscope as described previously (Yan *et al.*, 2012). Generally, three individuals (unless otherwise specified) of each species were collected randomly at each sampling time. For larval *C. idellus*, as well as the six individuals of juvenile, the full intestine of each individual was collected as a single sample, but 0.5 g of the foregut and hindgut contents were collected as different samples for each of the six adult individuals. However, for the carnivorous *S. chuatsi*, and *S. meridionalis*, including larvae and adults, individual stomach and hindgut samples were always analyzed separately. One exception was *S. meridionalis* at 3 dph, which we only collected the digestive tract of each individual as a single sample. Therefore, we obtained 58, 42, and 45 samples of *C. idellus*, *S. chuatsi*, and *S. meridionalis*, respectively (for further details, see Supporting Information Fig. S1). Genomic DNA was extracted using the PowerFecal® (gut samples) or PowerWater® (water samples) DNA Isolation Kit (Mo Bio, CA, USA) following the manufacturer's instructions. DNA concentrations and quality levels were determined using a ND-1000 spectrophotometer (NanoDrop, DE, USA), and all of the samples were diluted to the same concentration (2 ng μl^{-1}) for subsequent PCR amplification.

PCR amplification and MiSeq sequencing

The 16S rRNA gene was amplified following previously described methods (Wu *et al.*, 2015, Yan *et al.*, 2015). In brief, the 515f/806r primer set was used to amplify the V4 region of the 16S rRNA gene for analyzing gut microbiota. Each sample was amplified in triplicate in a reaction volume of 25 μl containing 1x PCR buffer, 0.4 μM of each primer, 0.5 U AccuPrime™ Taq (Invitrogen, CA, USA), and 10 ng genomic DNA using the following program: 1 min at 94°C, 10 cycles of 20 sec at 94°C, 25 sec at 53°C, and 45 sec at 68°C followed by a post-amplification extension of 10 min at 68°C. PCR products were purified using Agencourt® Ampure® XP beads (Beckman, CA, USA) according to the manufacturer's instructions. The purified DNA was then used as a template to perform a second PCR amplification using the same primer sequences and the protocol described above; however, 20 cycles were employed and sequencing adapters and barcodes were added for the identification of samples following Wu *et al.* (2015). Negative controls were performed each time to ensure that no contamination had occurred, and the three replicate amplifications for each sample were pooled for subsequent sequencing.

PCR products were quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, CA, USA). Equal amounts of each sample were combined, gel purified using a QIAquick Gel Extraction Kit (Qiagen, CA, USA), and then re-quantified using PicoGreen. The prepared DNA library was then sequenced at the Institute for Environmental Genomics in the University of Oklahoma using the MiSeq platform (Illumina, CA, USA) with 2 x 250 bp kits following the manufacturer's instructions. Quality filtering and processing of sequence reads were conducted on the Galaxy pipeline (http://zhoulab5.rccc.ou.edu:8080/root) as described previously (Wu *et al.*, 2015; Yan *et al.*, 2015). Preprocessed Illumina MiSeq sequences of fish gut bacteria are publicly available from the MG-RAST system (http://metagenomics.anl.gov/, project ID: 4626206.3). An OTU table was generated using the UPARSE clustering method (97% cutoff). To correct for differences in sequencing depth that may have affected the diversity evaluation (Fierer *et al.*, 2012), all samples for the same fish species were rarefied to the same sequencing depth by resampling OTUs prior to downstream analysis. In total, 10,316, 11,734 and 10,881 high quality sequences/sample were rarefied for the *C. idellus, S. chuatsi* and *S. meridionalis* libraries, respectively.

Phylogenetic analysis

Representative sequences of each OTU were aligned with a core set of 16S GreenGene reference sequences using PyNAST (Caporaso *et al.*, 2010), and high quality alignments

were then used to construct a maximum-likelihood tree using FastTree (Price *et al.*, 2009) for further phylogenetic analysis. As the significant phylogenetic signals (Mantel correlation, Pearson's *r*, $P < 0.05$) extended across relatively short distances (Supporting Information Fig. S6), it was most appropriate to quantify phylogenetic turnover among the closest relatives (Stegen *et al.*, 2012). So in the present study, we used the mean nearest taxon distance (MNTD) measure to determine which processes govern the assembly of microbiota in the fish gut. In addition, as microbial communities often include few high-abundance members coupled with a long tail of low abundance members (Shafquat *et al.*, 2014; Sogin *et al.*, 2006), we only focused on the weighted-abundance dataset in the following phylogenetic analysis.

In quantifying the phylogenetic diversity of a community within single samples, we calculated the weighted MNTD separated OTUs in a particular community according the following formula:

$$\mathrm{MNTD} = \sum_{i_k=1}^{n_k} f_{ik} \min\ (\triangle_{ikjk})$$

where f_{ik} is the relative abundance of OTU *i* in community *k*, n_k is the number of OTUs in community *k*, and min ($\triangle_{ikjk}$) is the minimum phylogenetic distance between OTU *i* and all other OTUs *j* in community *k*. The obtained standardized effect size measure (ses.MNTD), which is also known as the negative nearest taxon index (NTI, Webb *et al.*, 2002), can be used to determine ecological processes that govern a community in terms of phylogenetic structures as described previously (Stegen *et al.*, 2012; Wang *et al.*, 2013). Negative ses.MNTD values with low quantiles ($P < 0.05$) indicate that co-occurring species are more closely related than expected by chance (i.e., phylogenetic clustering). By contrast, positive ses.MNTD values with high quantiles ($P > 0.95$) suggest an overdispersion of co-occurring species (Webb *et al.*, 2002; Wang *et al.*, 2013). Moreover, a larger absolute magnitude of ses.MNTD value reflects greater effects of deterministic processes (Wang *et al.*, 2013).

Similarly, we also calculated the βMNTD between a given pair of samples as follows:

$$\beta\mathrm{MNTD} = 0.5\left[\sum_{i_k=1}^{n_k} f_{ik} \min\ (\triangle_{ikjk}) + \sum_{i_m=1}^{n_m} f_{im} \min\ (\triangle_{imjk})\right]$$

where min ($\triangle_{imjk}$) is the minimum phylogenetic distance between OTU *i* in community *k* and all OTUs *j* in community *m* (see above for other variables). The calculated βMNTDs, which reflect the dissimilarity between communities, were plotted along the fish developmental day-intervals to show the variations of gut microbiota. The difference between observed βMNTD and the mean of the null distribution is referred as βNTI. The βNTI in combinations of the Bray-Curtis-based Raup-Crick (RC_{bray}, Stegen *et al.*, 2013) was further used to quantify the contribution of major ecological processes that determine the assembly of fish gut microbiota according to methods as described in Stegen *et al.* (2013; 2015).

The relative influence of community turnover that is determined by homogeneous and variable selection (Vellend, 2010) is denoted by $\beta\mathrm{NTI} < -2$ and $\beta\mathrm{NTI} > +2$ fractions, respectively. Variable selection results in community to be different due to the differences in selective environments among local scales, whereas homogeneous selection results in community composition to be similar due to a consistent selective environment among local scales (Martínez *et al.*, 2015). If $|\beta\mathrm{NTI}| < 2$ but $RC_{bray} > +0.95$ or < -0.95, community turnover is governed by dispersal limitation or homogenizing dispersal processes (Stegen *et al.*, 2013), respectively. Dispersal limitation results in divergence in community composition due to limited exchange, whereas homogenizing dispersal results in community composition to be similar among local scales due to dispersal. However, if $|\beta\mathrm{NTI}| < 2$ and $|RC_{bray}| < 0.95$, then drift (referred to as 'undominated' processes in Stegen *et al.* 2015) drives compositional turnover processes. Drift was used to estimate the fraction that neither selection nor dispersal is the primary cause of between-community compositional differences (Martínez *et al.*, 2015).

Statistical analysis

The rarefied OTU tables were further analyzed using the following statistical methods: (i) alpha- and beta-diversity comparisons were conducted to reveal changes in gut microbiota throughout host development; (ii) UniFrac distance-based PCoA analysis and taxonomic composition-based DCA ordination were conducted to illustrate overall patterns of microbial communities throughout host development; (iii) non-parametric tests including multiple-response permutation procedure (MRPP) and permutational multivariate analysis of variance (PERMANOVA) tests were conducted to compare community dissimilarities with Bray-Curtis distances (Zhou *et al.*, 2014); (iv) significance tests were performed through an analysis of variance (ANOVA) with least-significant-difference (LSD) to examine whether differences between host stages were significant or not; (v) a null model analysis involving a permutational analysis of multivariate dispersion (PERMDISP) (Chase *et al.*, 2011) was performed to reveal whether the observed β-diversity value is indistinguishable from the null expectation. All statistics were performed using the 'vegan', 'phyloseq' and 'picante' R programs (R Foundation for Statistical Computing, Vienna, Austria).

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Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Experimental design showing important information during the course of the study. The number of samples collected at the corresponding time point (indicated by the symbol of '♦') is shown. The information of diet and which environment they are from is also given.

Fig. S2. The gut Proteobacteria alpha-diversity (A–C) and the weighted standardized effect size of the mean nearest taxon distance (ses.MNTD) (D–F) were significantly correlated with the host developmental day (all P values < 0.05).
Fig. S3. The gut Firmicutes alpha-diversity (A-C) and the weighted standardized effect size of the mean nearest taxon distance (ses.MNTD) (D-F) were significantly correlated with the host developmental day (all P values < 0.05).
Fig. S4. Relative abundance of the dominant microbial phyla detected in the guts of fish at different developmental stages. Stages were assigned consistent with the gut microbiotas with significant differences (see Table 1). Mean values of samples collected from each stage were plotted with the SEs. The variations across stages were tested through an ANOVA, with the presence of different letters denoting significant differences between stages from least-significant-difference (LSD) tests and with the presence of the same letter denoting no significant difference in abundance.
Fig. S5. The relative abundance of the dominant microbial phyla detected in the guts of adult fish. Mean values of samples collected from each species were plotted with the SEs. Species identities are as follows: CI: *Ctenopharyngodon idellus*, CM: *Carnis megalobramae*, CA: *Carassius auratus*, CC: *Cyprinus carpio*, SC: *Siniperca chuatsi*, CM: *Channa micropeltes*, SM: *Silurus meridionalis*.
Fig. S6. The Mantel correlation between the pairwise matrix of OTU niche distances and the phylogenetic distances for each fish library with 999 permutations. Significant correlations ($P < 0.05$) of phylogenetic signals in species ecological niches are denoted as solid circles, and un-shaded circles denote non-significant correlations.
Fig. S7. The relationships between weighted beta mean nearest taxon distances (βMNTD) and host developmental day-intervals were significant (all P values < 0.05).
Fig. S8. Summary of the contribution of the ecological processes that determine community assembly of gut microbiota in seven of the investigated fish species at adult stage. Species identities are as follows: CI: *Ctenopharyngodon idellus*, CM: *Carnis megalobramae*, CA: *Carassius auratus*, CC: *Cyprinus carpio*, SC: *Siniperca chuatsi*, CM: *Channa micropeltes*, SM: *Silurus meridionalis*.
Table S1. Relative abundance of the dominant bacterial genera (mean abundance >1%) in the guts of *Ctenopharyngodon idellus* (A), *Siniperca chuatsi* (B) and *Silurus meridionalis* (C). Numbers in cells denote percentages of relative abundance. The highest abundances are shown in red, and the lowest values are shown in white. Variations between different stages were tested through an ANOVA. The presence of different letters denotes significant differences between stages from least-significant-difference (LSD) tests, and the presence of the same letter denotes no significant difference in abundance.
Table S2. Bray-Curtis distance-based dissimilarity test showing differences between microbial communities in the water used to rear larval *Ctenopharyngodon idellus in situ*.