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Supplemental Data

Reciprocal Gut Microbiota Transplants from Zebrafish and Mice to Germ-free Recipients Reveal Host Habitat Selection

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Supplemental Experimental Procedures

16S rRNA Gene Sequencing

Luminal contents from the ceca of CONV-R adult mice and Z-mice, and the intact digestive tracts of CONV-R adult zebrafish and 6dpf/10dpf M-zebrafish were removed immediately after animals were killed, and homogenized in sterile PBS under aerobic conditions (see **Fig. S1, Table S1**). An aliquot of each homogenate was used immediately for culture-based enumeration (see below): the remainder was frozen at –80^{o}C until use.

A frozen aliquot of each sample was thawed, centrifuged at 18,000 x g for 30 min at 4^{o}C to pellet material, and the pellet was then pulverized with a sterile pestle in 700µL filter-sterilized extraction buffer [100mM NaCl, 10mM Tris-Cl (pH 8.0), 25mM EDTA (pH 8.0), 0.5% (w/v) SDS, 0.1 mg/mL proteinase K (Sigma)]. Following a 40 min incubation at 37^{o}C for 40 min, 500µL of 0.1mm-diamter zirconia/silica beads (Biospec Products) plus 500µL of a mixture of phenol:chloroform (Ambion) were added to each sample, and the sample was disrupted mechanically for 2 min at 23^{o}C with a bead beater (Mini-Beadbeater, BioSpec Products Inc.; using the instrument's highest setting). Samples were centrifuged at 18,000 x g at 4^{o}C for 3 min. The aqueous phase was subjected to one additional round of phenol:chlorofom extraction prior to precipitation of DNA with isopropanol. Isolated genomic DNA was further purified over Montage PCR Centrifugal Filters (Millipore).

For each sample, three replicate 25µL polymerase chain reactions were performed, each containing 1-200 ng of purified genomic DNA, 20mM Tris-HCl (pH 8.4), 50mM KCl, 300uM $MgCl_2$, 400mM Betaine, 160µM dNTPs, 3 units of Taq DNA polymerase (Invitrogen), and 400nM of universal 16S rRNA primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1491R (5'-GGTTACCTTGTTACGACTT-3'). Reactions were incubated initially at 94^{o}C for 10 min, followed by 30 cycles of 94^{o}C for 1 min, 52^{o}C for 1 min, and 72^{o}C for 2 min, and a final extension step at 72^{o}C for 10 min. Replicate reactions were pooled and purified over Montage PCR Centrifugal Filters (Millipore), and pooled PCR products cloned into pCR4-TOPO (TOPO TA Cloning Kit for Sequencing, Invitrogen). DNA extraction of control samples from GF animals did not yield detectable 16S rRNA PCR products or colonies. Clones were sequenced in BigDye Terminator reactions using 16S rRNA primers [27F, 1491R, and 907R (5'-CCGTCAATTCCTTTRAGTTT-3')]. 16S rRNA sequences derived from these culture-independent surveys were submitted to GenBank under accession numbers DQ813844-DQ819370.

Culture-Based Enumerations

To recover culturable bacteria from microbial consortia, homogenates of pooled zebrafish digestive tracts or individual mouse ceca were plated under aerobic conditions in a dilutional series on BHI-blood agar, tryptic soy agar, PEA-blood agar, nutrient agar, marine agar, and cholera agar (Becton Dickinson), and grown at 37^{o}C and/or 28.5^{o}C under aerobic and anaerobic conditions. Colonies were picked in a non-random manner into the corresponding liquid media under aerobic conditions, and grown under the same conditions that led to their initial detection. Liquid cultures were frozen as glycerol stocks in 96-well microtiter plates.

Aliquots (1μL) of these glycerol stocks were used directly as templates for 25μL PCR with the 27F and 1491R primers described above. PCR products were purified over Perfectprep PCR Cleanup 96-well plates (Eppendorf), and partial 16S rRNA sequences were generated using 27F primer. The resulting 16S rRNA sequences with ≥700 Phred >Q20 bp were aligned in Arb and analyzed as described above. These 575 16S rRNA sequences are available on our lab website at http://gordonlab.wustl.edu/supplemental/Rawls/Cultured_Clone_Seqs_FastA.txt. A subset of these cultured clones were subsequently recovered from glycerol stocks and re-sequenced using both 27F and 1491R primers to confirm their identity and to provide more complete sequence coverage (GenBank accession numbers DQ819371-DQ819377).

Functional Genomics

To compare gene expression in zebrafish reared under different conditions, two biological duplicate pools of animals from each group were analyzed. For zebrafish reared in gnotobiotic isolators, digestive tracts were removed *en bloc* under a dissecting microscope and pooled (n= 10-40/pool). For zebrafish reared in tissue culture flasks, intact larvae were pooled (n= 6-17/pool). Each pooled collection was homogenized by repeated passage through a 20-gauge needle, and total RNA was then extracted (TRIzol reagent; Invitrogen).

To compare gene expression in mice reared under different conditions, 3-5 animals were analyzed per treatment group. Immediately after each animal was killed, its small intestine was removed, divided into 16 equal-size segments, and segment 14 (ileal sample) was taken. The segment was homogenized, and RNA was extracted (Rneasy Miniprep Kit; Qiagen).

The quantity and quality of zebrafish and mouse gut RNA were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies). RNA preparations were then used as templates for generating cDNAs (Superscript II reverse transcriptase; random primers; Invitrogen).

qRT-PCR assays were performed as described (Rawls et al., 2004), except that each 25μL reaction mixture contained cDNA corresponding to 2ng of total RNA from zebrafish digestive tracts, plus 900 nM gene-specific primers (except zebrafish 18S rRNA-specific control primers which were used at 300 nM) (**Table S16**). Assays were performed in triplicate using Absolute SYBR Green ROX Mix (ABgene) and a MX3000P QPCR Instrument (Stratagene). Data were normalized to 18S rRNA ($\Delta\Delta C_T$ analysis).

Whole genome transcriptional profiling was performed using Affymetrix GeneChips. cRNA targets were prepared, and hybridized (40 μg/sample) to 430 v2 mouse GeneChips using established protocols (Hooper et al., 2001). CEL files were normalized using RMA (Bolstad, 2004; http://rmaexpress.bmbolstad.com/), and all probesets with an average intensity across all arrays >50 were analyzed using Significance Analysis of Microarrays software (SAM version 2.21; Tusher et al., 2001). For M-mice vs. GF mice and Z-mice vs. GF mice comparisons, a false-discovery rate of <1% and a post-analysis fold-change cut-off of ≥1.5 were used, and all genes with ≥50% present calls (calculated using Affymetrix Microarray Suite 5.0) across all

replicate experimental or reference arrays were culled for further analysis. The resulting datasets were analyzed using the Ingenuity Pathways Analysis (IPA) software tool (http://www.ingenuity.com) according to Giannakis et al. (2006). IPA annotations take into account Gene Ontology (GO) annotations, but are distinct and based on a proprietary knowledge base of over 1,000,000 protein-protein interactions. The IPA output includes metabolic and signaling pathways: statistical assessments of the significance of their representation are based on a right-tailed Fisher's Exact Test, which is used to calculate the probability that genes participate in a given pathway relative to their occurrence in all other pathway annotations.

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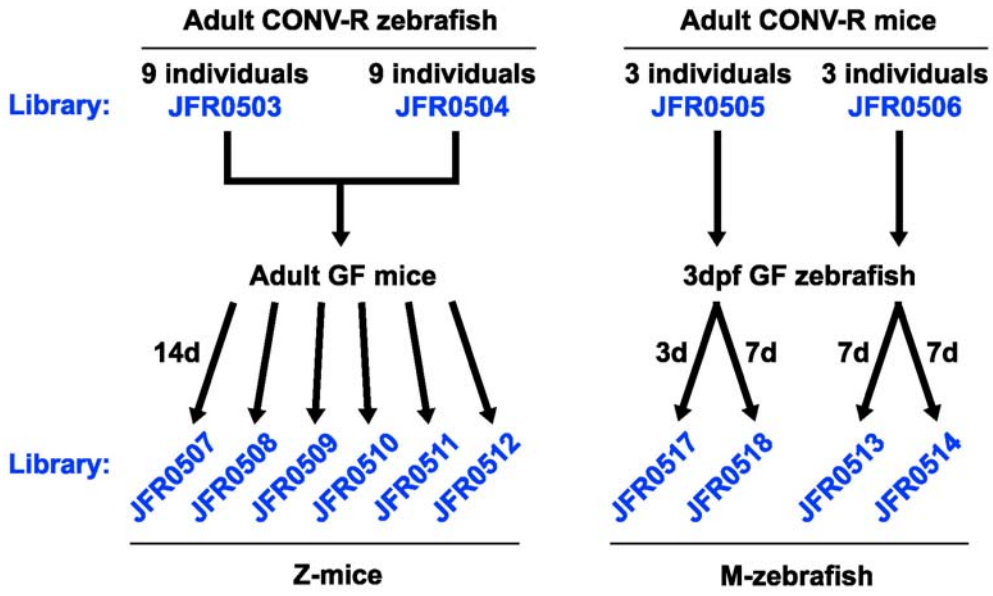

Figure S1. Flow Chart of Reciprocal Transplantation Experimental Design

The gut microbiota from two independent pools of 9 adult conventionally-raised (CONV-R) zebrafish were harvested, combined, and used to colonize 6 adult germ-free (GF) mice, yielding Z-mice. 14 days later, the cecal contents from individual Z-mice were harvested for analysis. The gut microbiota from 3 adult conventionally-raised mice were harvested and used to colonize 3dpf germ-free zebrafish, yielding M-zebrafish. 3 or 7 days later, the gut contents from groups of M-zebrafish were harvested. As indicated, this mouse-into-zebrafish experiment was performed in duplicate. Culture-independent 16S rRNA libraries generated from these different samples (Library) are indicated in blue text.

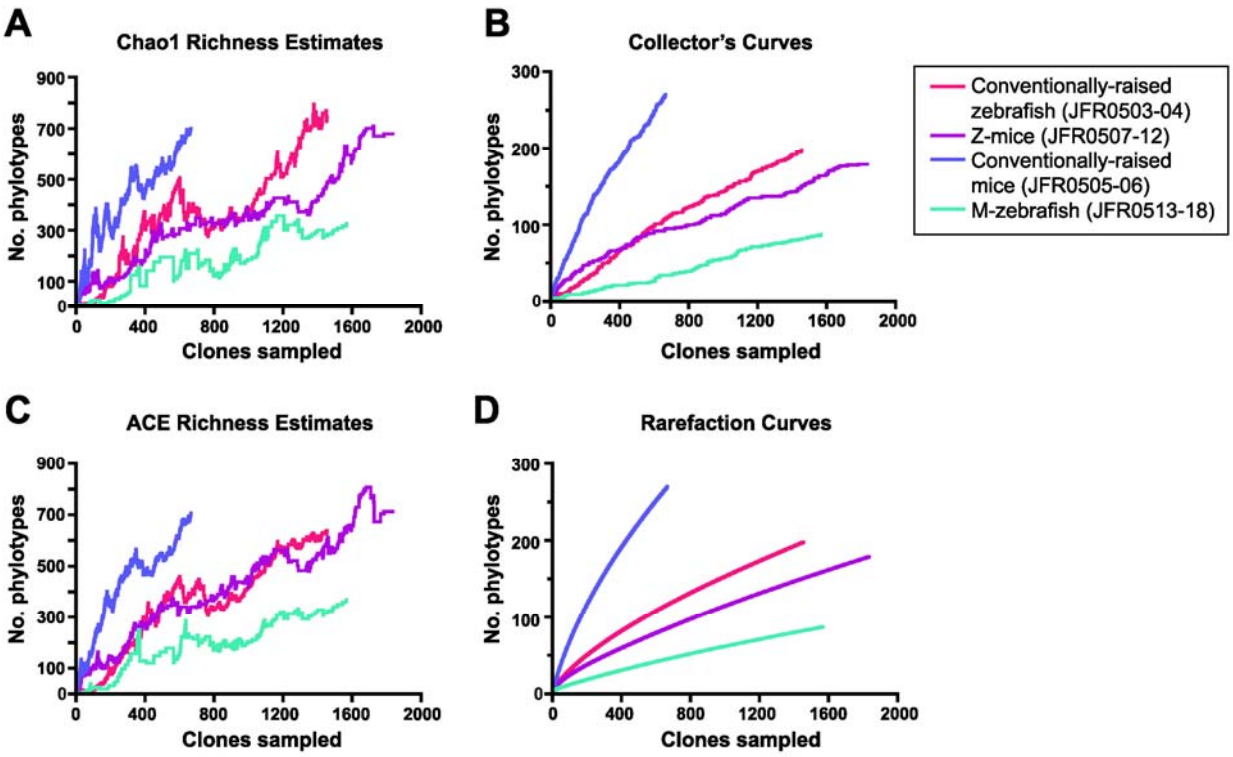

Figure S2. Sample-Based Assessments of Diversity and Coverage in Reciprocal Transplantation Experiments

The pooled libraries from the intestines of conventionally-raised zebrafish (CONV-R fish; libraries JFR0503-04), mice colonized with a zebrafish microbiota (Z-mice; libraries JFR0507-12), conventionally-raised mice (CONV-R mice; JFR0505-06), and zebrafish colonized with a normal mouse microbiota (M-zebrafish; JFR0513-18) were analyzed using DOTUR (Schloss and Handelsman, 2005). The phylotype richness for each treatment is expressed as full bias corrected Chao1 richness estimates (panel **A**) and abundance-based coverage estimates (ACE; panel **C**). The number of observed phylotypes (99%ID) and the number of sequences sampled are shown as Collector's curves (panel **B**) and Rarefaction curves (panel **D**). The addition of clones along the X-axis is non-random (ordered by library), producing the variability seen in panels **A** and **C**.

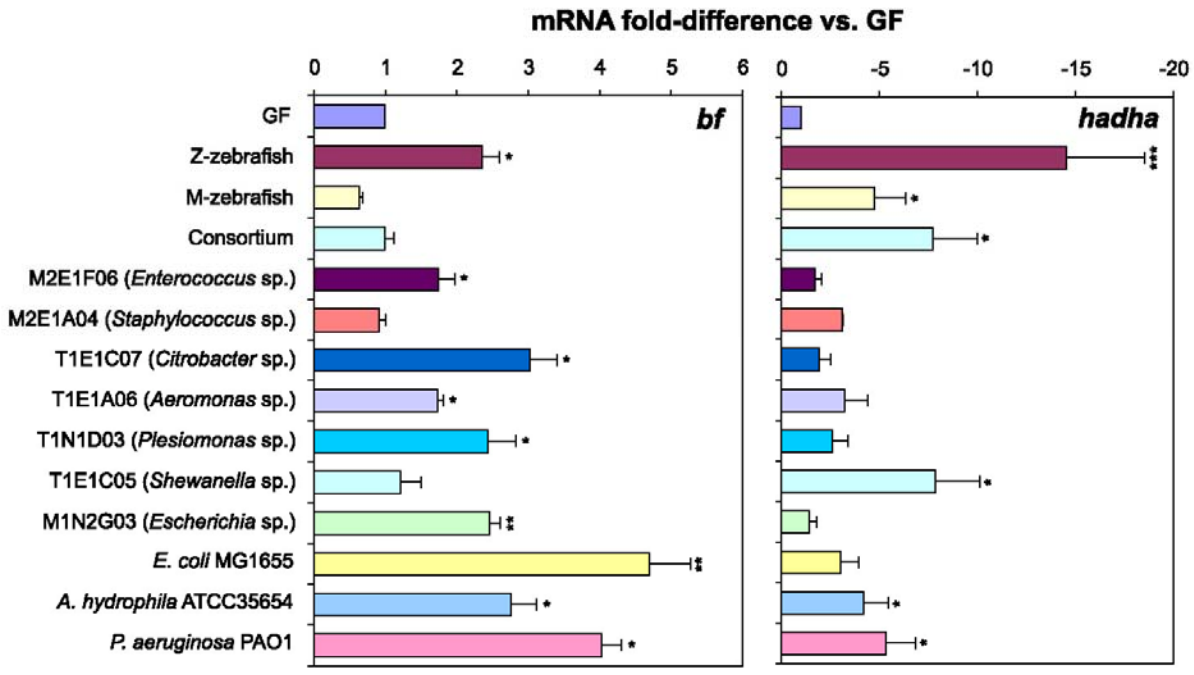

Figure S3. qRT-PCR Assays of the Responses of Germ-free Zebrafish to Colonization with Individual Culturable Members of the Zebrafish and Mouse Gut Microbiota

Expression levels of *complement factor b* (*bf*) and *hydroxyacylCoA dehydrogenase/3-ketoacylCoA thiolase/enoyl CoA hydratase* (*hadha*) were assessed using RNA extracted from the pooled digestive tracts of 6dpf zebrafish inoculated since 3dpf with a CONV-R zebrafish microbiota (Z-zebrafish), a CONV-R mouse microbiota (M-zebrafish), a consortium of 7 primary isolates (Consortium), a primary *Enterococcus* isolate (M2E1F06), a primary *Staphylococcus* isolate (M2E1A04), a primary *Citrobacter* isolate (T1E1C07), a primary *Aeromonas* isolate (T1E1A06), a primary *Plesiomonas* isolate (T1N1D03), a primary *Shewanella* isolate (T1E1C05), a primary *Escherichia* isolate (M1N2G03), an *Escherichia coli* type strain (MG1655), an *Aeromonas hydrophila* type strain (*A.hydrophila* ATCC35654), or a *Pseudomonas aeruginosa* type strain (PAO1). Data from biological duplicate pools (≥10 animals

per pool) were normalized to 18S rRNA levels and results are expressed as mean fold-change compared to GF controls ± SEM. ***, $P<0.0001$; **, $P<0.001$; *, $P<0.05$.

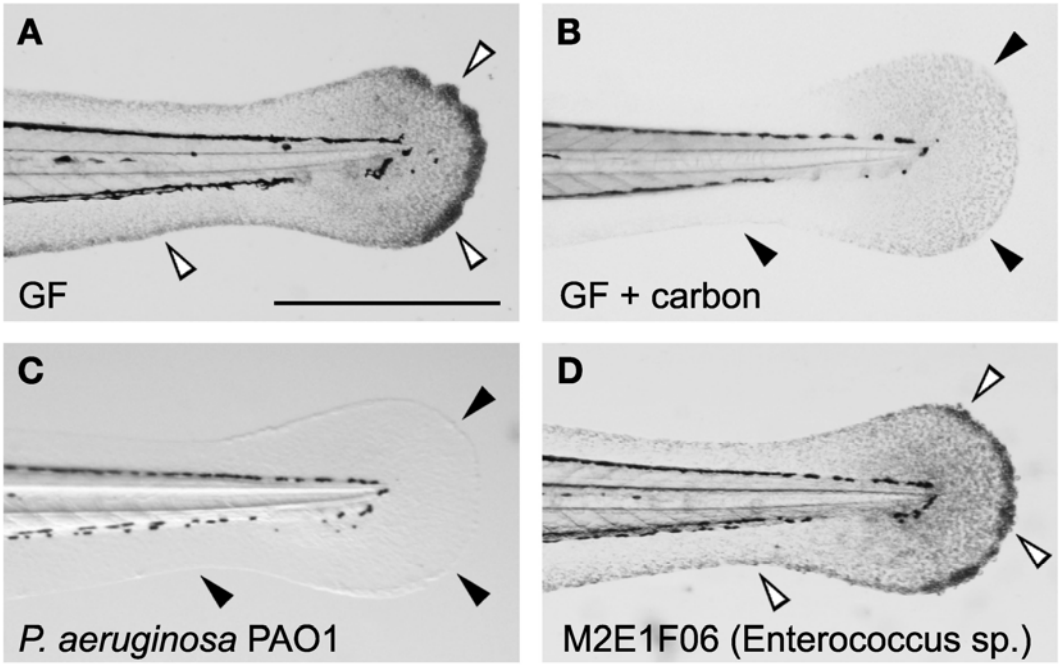

Figure S4. The Epidermal Degeneration Phenotype in Fed GF Zebrafish Is Ameliorated with Different Treatments

(**A**) Caudal region of a live 9dpf GF zebrafish, fed since 3dpf, displays loss of the transparency and integrity of the fin fold epidermis (white arrowheads; Rawls et al., 2004). (**B**) Age-matched fed GF zebrafish raised since 3dpf in the presence of activated carbon and ammonia-removing cation exchange resin (GF+carbon). The result is improved epidermal transparency and integrity (black arrowheads). GF zebrafish can survive under these conditions beyond 30dpf (data not shown). **(C)** 9dpf zebrafish colonized since 3dpf with *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa* PAO1) do not develop the epidermal phenotype, as indicated by the healthy transparent fin fold epithelium (black arrowheads). (**D**) In contrast, 9dpf larvae colonized since 3dpf with a primary *Enterococcus* isolate (M2E1F06) display a phenotype similar to GF controls (white arrowheads). Scale bar: 500μm.

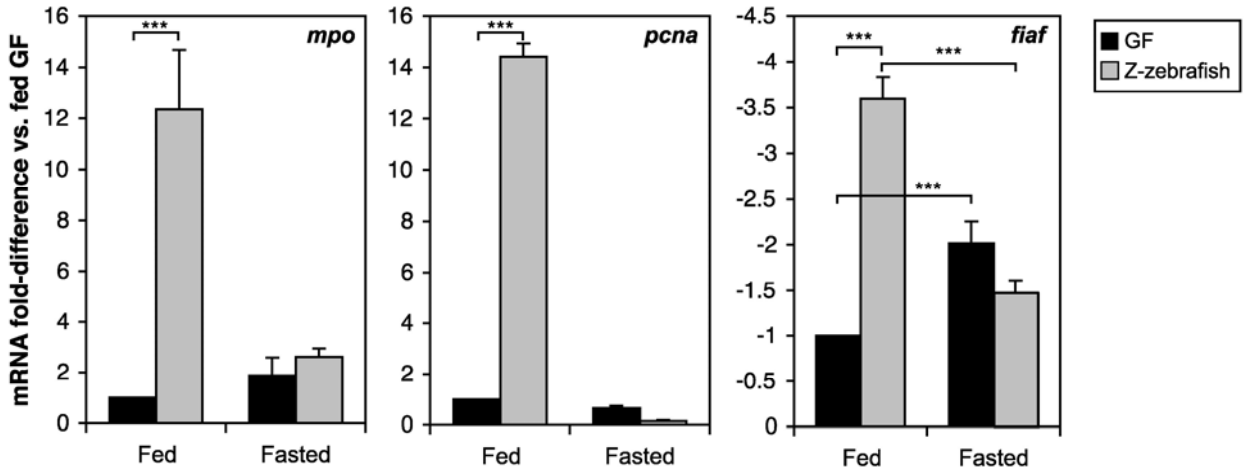

Figure S5. Zebrafish Host Responses to the Gut Microbiota Are Attenuated in the Absence of an Exogenous Nutrient Supply

6dpf zebrafish that were either germ-free (GF) or colonized since 3dpf with a CONV-R zebrafish microbiota (Z-zebrafish) and fed an autoclaved diet beginning at 3dpf (Fed) were compared with GF and Z-zebrafish siblings deprived of all food (Fasted). Expression levels of *myeloperoxidase* (*mpo*), *proliferating cell nuclear antigen* (*pcna*), and *fasting-induced adipose factor* (*fiaf*) were assessed by qRT-PCR using RNA extracted from the pooled digestive tracts of 6dpf zebrafish. Data from biological duplicate pools (≥10 animals per pool) were normalized to 18S rRNA levels and the results are expressed as mean fold-change compared to fed GF controls ± SEM. Note that nutrient (*fiaf*), innate immune (*mpo*) and proliferative responses (*pcna*) to colonization are markedly attenuated in fasted animals. Similar *fiaf* results were obtained in fed and fasted 6dpf zebrafish colonized with either *P. aeruginosa* PAO1 or *A. hydrophila* ATCC35654 since 3dpf (data not shown). Importantly, fasting did not produce a statistically significant reduction in gut microbial density in any of the colonization groups (data not shown). The sensitivity of 6dpf zebrafish to the presence of an exogenous nutrient supply was unanticipated: at this age, zebrafish have been consuming food for only 1-2 days; moreover, many 6dpf zebrafish have not

completed yolk resorption and, therefore, are presumably still utilizing this endogenous food source. ***, $P<0.0001$; **, $P<0.001$; *, $P<0.05$.