Short Communication

LARVAL EXPOSURE TO POLYCHLORINATED BIPHENYL 126 (PCB-126) CAUSES PERSISTENT ALTERATION OF THE AMPHIBIAN GUT MICROBIOTA

Kevin D. Kohl,*† Tawnya L. Cary,‡ William H. Karasov,§ and M. Denise Dearing†
†Department of Biology, University of Utah, Salt Lake City, Utah, USA
‡Department of Zoology, University of Wisconsin–Madison, Madison, Wisconsin, USA
§Department of Forest and Wildlife Ecology, University of Wisconsin–Madison, Madison, Wisconsin, USA

Abstract: Interactions between gut microbes and anthropogenic pollutants have been under study. The authors investigated the effects of larval exposure to polychlorinated biphenyl 126 (PCB-126) on the gut microbial communities of tadpoles and frogs. Frogs treated with PCBs exhibited increased species richness in the gut and harbored communities significantly enriched in Fusobacteria. These results suggest that anthropogenic pollutants alter gut microbial populations, which may have health and fitness consequences for hosts. *Environ Toxicol Chem* 2015;34:1113–1118. © 2015 SETAC

INTRODUCTION

As a group, amphibians are undergoing rapid extinctions and serve as important environmental sentinels to monitor ecosystem health [1]. Environmental pollutants may contribute to amphibian extinctions by altering aspects of amphibian physiology, such as decreasing the rate of development [2,3], immune function [4,5], or proper reproductive development [6]. The gut microbiota is another important physiological system that provides several services to the host [7]. However, the effects of environmental pollutants on these symbiotic communities are poorly understood. We recently conducted the first culture-independent microbial inventory of the gut microbiota of an amphibian and demonstrated that amphibians undergo a large restructuring of the microbiota during metamorphosis [8]. In the present study, we investigate the effects of environmental pollutants on the amphibian gut microbiota.

Polychlorinated biphenyls (PCBs) represent some of the most widespread anthropogenic contaminants. These compounds were used heavily as commercial flame retardants and insulating fluids through the mid-20th century. Many of these compounds persist in the environment and bioaccumulate in the tissues of wild amphibians [9]. Polychlorinated biphenyls compromise the immune function of a wide array of vertebrate taxa [10–12] and have been shown to alter the gut microbial community structure in mice [13]. Altering the gut microbial community may impact the performance and fitness of the host due to the numerous functions the microbiota perform.

In the present study, we used northern leopard frogs (*Lithobates pipiens,* also known as *Rana pipiens*) as a model ecological species to study the effects of PCBs on gut microbial communities. We inventoried the communities of tadpoles and frogs following larval exposure to PCB-126 to examine the possibility that environmental pollutants alter gut microbial ecology. Demonstrating these effects would provide evidence that the gut microbiota may be susceptible to environmental pollutants.

MATERIAL AND METHODS

Animals and sample collection

Samples for the present study were collected from a larger study investigating the effects of PCB-126 on the performance and survival of Northern leopard frogs (*L. pipiens*). Embryos were purchased from a laboratory colony at Nasco, transferred to the laboratory, and allowed to hatch without manipulation. Six days post fertilization (dpf), tadpoles were free-swimming (Gosner Stage 25) [14], and were distributed into 8 control glass aquaria and 5 PCB-exposed aquaria. Aquaria were filled with 13 L of dechlorinated, filtered, ultraviolet-irradiated tap water. Water changes were performed 4 times per week to provide standard water quality (pH = 7.8–8.3, nitrite < 1.0 mg/L, total NH_3 < 1 mg/L, hardness ~180 mg/L). In addition, air stones in each aquarium provided sufficient aeration throughout the experiment (dissolved oxygen ≥ 50% saturation). Conditions of the animal facility were a 14:10-h light:dark:light cycle, $23 \pm 1\,°C$ water temperature, and humidity ≥ 30%. Tadpoles were fed ad libitum a diet of ground rabbit chow (Harlan Teklad; Cat 2030; 250 g/L) mixed with agar (20 g/L), gelatin (14 g/L), and 0.2 μm filtered, dechlorinated water. The mixture was brought to a boil for 1 min and then cooled to room temperature. Tadpoles were fed either diets containing 0 (control) or 7.3 ng PCB-126/g wet mass (chemical analyses were performed using US Environmental Protection Agency [USEPA] extraction method 3541 and USEPA method 8082 A; ALS Environmental). Tadpoles for the present study were a subset of the larger study, and were collected from 4 control aquaria and 2 PCB-exposed aquaria when they reached 91 dpf (85 d of PCB-126 exposure) and were at similar developmental points (Gosner Stage = 36.1 ± 1.8). Tadpoles were euthanized in 1% buffered tricaine methanesulfonate, and digesta was collected and frozen (control fed tadpoles: *n* = 7; PCB-126 fed tadpoles: *n* = 7). For analysis of frog gut microbiota, tadpoles were allowed to develop through metamorphosis and housed individually in 0.5-L polypropylene jars. Twenty-five milliliters of dechlorinated,

filtered water was added to the jars, and the jars were tilted to provide both a wet and dry surface for the frogs. Frogs were fed an uncontaminated diet of crickets and mealworms 6 times per week, supplemented with calcium and vitamin powder for 16 wk. Thus, the frogs were not exposed to dietary PCBs for 16 wk. At 16 wk postmetamorphosis, frogs were euthanized in 1% buffered tricaine methanesulfonate, and digesta was collected and frozen (control diet as tadpoles: $n = 8$; PCB-126 diet as tadpoles: $n = 8$). Frogs were, on average, 185.7 ± 2.4 d old at this point and had been exposed to PCB-126 as tadpoles for an average duration of 61.3 ± 3.6 d. The University of Wisconsin's Institutional Animal Care and Use Committee approved all experimental procedures involving tadpoles and juvenile frogs.

Microbial inventories

Whole DNA was isolated from gut contents using a QIAamp DNA Stool Mini Kit, which was then sent to Argonne National Laboratory to undergo sequencing. Microbial inventories were conducted by amplifying the V4 region of the 16 S rRNA gene using primers 515F and 806R and paired end sequencing on an Illumina MiSeq platform [15]. Sequences were analyzed using the QIIME software package [16]. Sequences underwent standard quality control and were split into libraries using default parameters in QIIME. Sequences were grouped into operational taxonomic units using UCLUST [17] with a minimum sequence identity of 97%. The most abundant sequences within each operational taxonomic unit were designated as a "representative sequence," and then aligned against the Greengenes core set [18] using PyNAST [19], with default parameters set by QIIME. A PH Lane mask (QIIME) was used to remove hypervariable regions from aligned sequences. FastTree [20] was used to create a phylogenetic tree of all representative sequences. Operational taxonomic units were classified using the Ribosomal Database Project classifier with a standard minimum support threshold of 80% [21]. Sequences identified as chloroplasts or mitochondria were removed from analysis.

We then compared the relative abundances of microbial taxa. Relative abundances of microbial phyla and genera were normalized using variance stabilizing transformation of arcsin-(abundance$^{0.5}$) [22,23]. Because the microbial community is largely restructured between tadpoles and frogs [8], the present study focuses on the effect of PCBs within a life stage. Transformed abundances were compared using *t*-tests within each life stage, using the False Discovery Rate correction for multiple comparisons. For microbial phyla, we compared the abundances of the 5 most abundant phyla, and for microbial genera, we compared all genera that were present in at least 2 samples. We used a Pearson chi-square to test for differences in prevalence or the proportion of amphibian individuals hosting a certain taxa. For all statistical tests we used an α value of 0.05.

We calculated several measurements of diversity. First, we calculated Faith's phylogenetic diversity [24], which randomly samples several operational taxonomic units from each sample and measures the cumulative branch lengths from the phylogenetic tree of all sequences. We also calculated an estimate of species richness (Chao1). For these measurements, we calculated the mean of 20 iterations for a subsampling of 9500 sequences from each sample. We compared diversity metrics using a nested analysis of variance with life stage and PCB exposure as variables and tanks nested within PCB exposure treatments. In addition, we investigated potential covariates of age and Gosner stage for tadpoles. Insignificant covariates were removed from the final analysis.

We also calculated unweighted UniFrac distances, which are the fractions of total branch lengths unshared between 2 samples on the phylogenetic tree of all samples [25]. We visualized similarities and differences in overall microbial community composition across groups using Principal Coordinates Analysis of unweighted UniFrac distances. Community membership was statistically compared using adonis, a permutational multivariate analysis of variance that uses distance matrices to investigate sources of variation [26]. All sequences were deposited in the Sequence Read Archive under accession SRP019766.

RESULTS

At 16 wk postmetamorphosis, frogs fed the 7.3 ng PCB-126/g diet had mean body burden residues of 3.38 ng PCB-126/g body mass, whereas control animals had a body burden of 0.07 ng/g body mass.
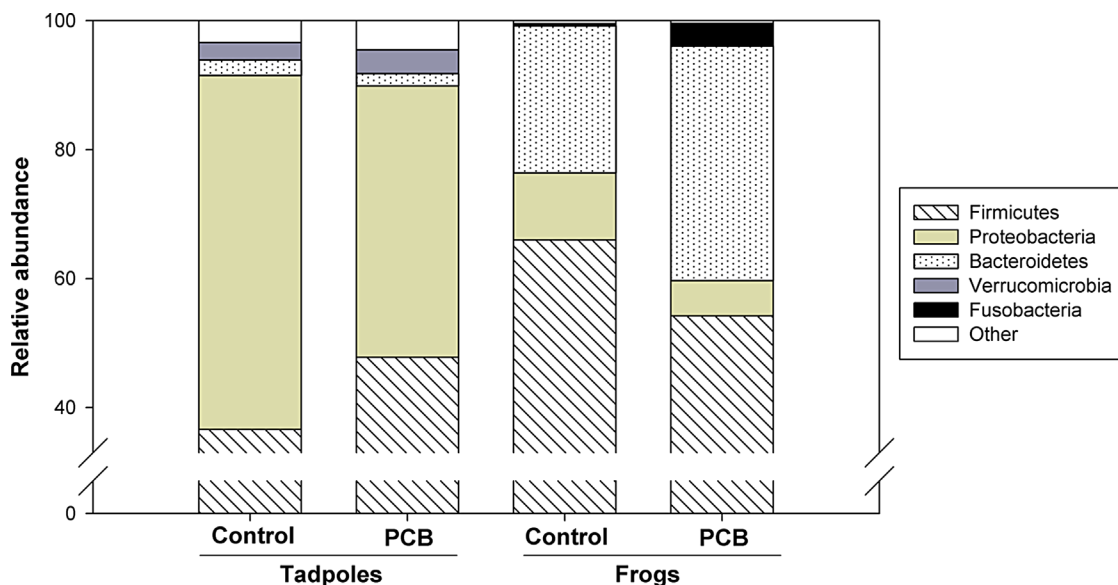

Figure 1. Relative abundances of dominant microbial phyla in the guts of control and polychlorinated biphenyl-treated (PCB) tadpoles and frogs.

Our sequencing effort resulted in a total of 462 947 high quality microbial 16 S rRNA sequences (average of 13 930 ± 403 sequences per sample). These sequences were classified into 7908 operational taxonomic units based on 97% sequence identity.

We observed modest differences in the gut microbial community structure of tadpoles due to larval exposure to PCB-126. As tadpoles, no bacterial phyla or genera that satisfied the statistical requirements to test for differences exhibited significantly different relative abundances between control and PCB-treated tadpoles. Some bacteria genera exhibited differences in prevalence, such that 6 of the 7 control tadpoles harbored *Aminobacter*, whereas this genus was absent from the guts of all PCB-treated tadpoles ($\chi^2(1, N = 14) = 10.5$; $p = 0.0012$). Similarly, *Pseudomonas* was only detected in 1 of the 7 control tadpoles and was present in all 7 PCB-treated tadpoles ($\chi^2(1, N = 14) = 10.5$; $p = 0.0012$).

Interestingly, we observed a lasting effect of larval exposure to PCB-126 on the relative abundances of microbes residing in the guts of frogs. Frogs that were exposed to PCB-126 as tadpoles maintained a higher abundance of Fusobacteria ($t = 2.95$; $df = 14$; $p = 0.01$; Figure 1). The phylum Fusobacteria comprised a very small portion of the tadpole (average of control and PCB-treated: 0.008%) and control frog (0.3 ± 0.1%) gut communities. However, frogs that underwent larval exposure to PCB-126 harbored a community with 3.5 ± 1.4% relative abundance of Fusobacteria, with some individuals maintaining a community comprised of >10% Fusobacteria. The operational taxonomic unit that drove this response is an uncultured member of the genus *Fusobacterium*, as determined by BLAST analysis. This difference represents a lasting effect of larval PCB-126 exposure, as postmetamorphic frogs were not subjected to dietary PCB-126. At the genus level, we did not detect any bacteria with differential abundance or prevalence between control and PCB-exposed frogs.

Diversity metrics differed as a result of larval PCB-126 exposure. Individuals treated with PCB exhibited increased phylogenetic diversity harbored within the gut, as well as an
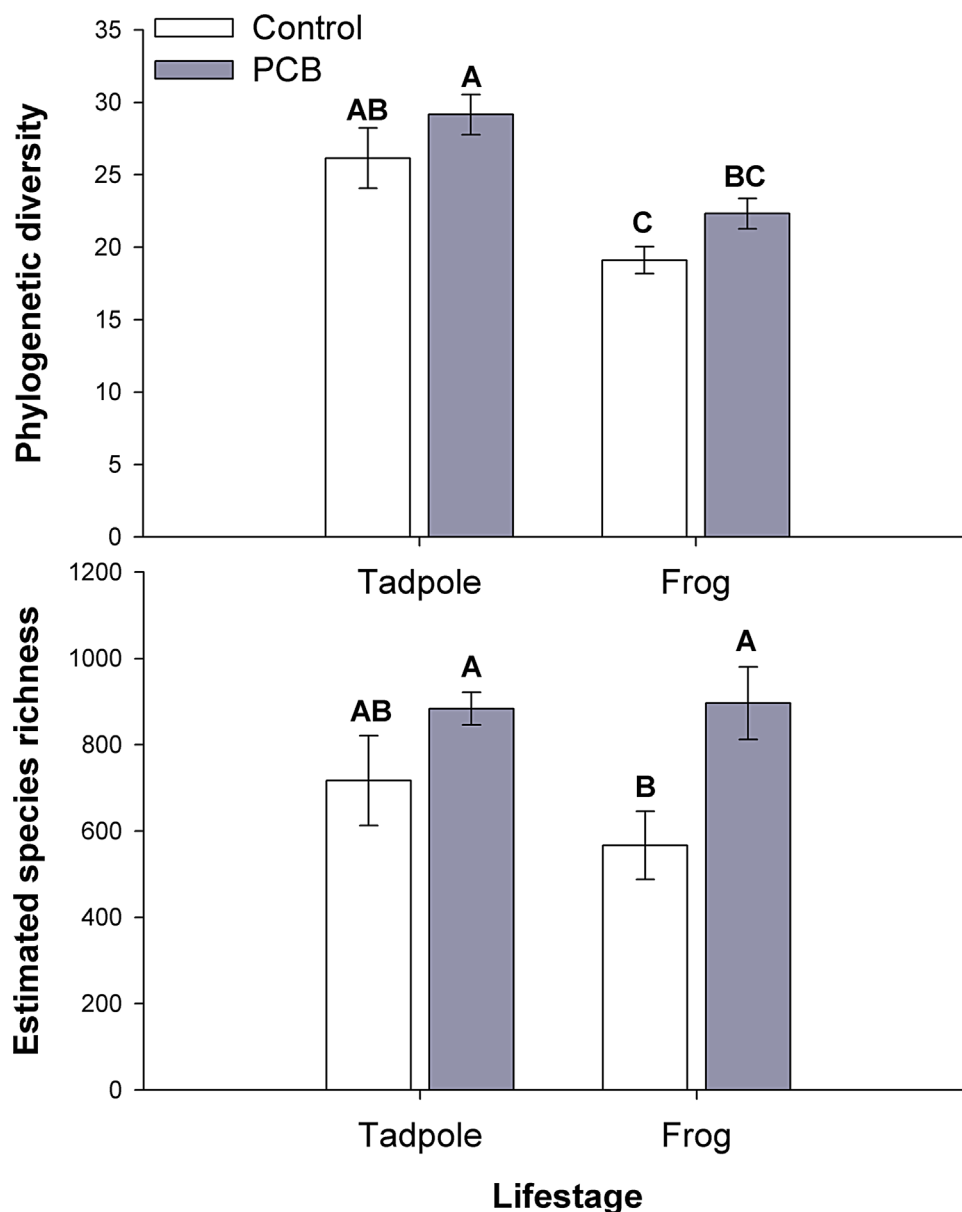

Figure 2. Estimated species richness and phylogenetic diversity of control and polychlorinated biphenyl-treated (PCB) tadpoles and frogs. Different letters above the bars correspond to significant differences using Tukey's Honest Significant Difference test.

Table 1. Summary of analysis of variance results for alpha diversity metrics[a]

Diversity index	F	P
Phylogenetic diversity		
Life stage	12.24	0.003
Polychlorinated biphenyl exposure	4.26	0.05
Life stage × polychlorinated biphenyl exposure	2.65	0.12
Tank nested in polychlorinated biphenyl exposure	1.38	0.27
Estimated species richness		
Life stage	0.56	0.46
Polychlorinated biphenyl exposure	11.88	0.003
Life stage × polychlorinated biphenyl exposure	1.76	0.20
Tank nested in polychlorinated biphenyl exposure	1.53	0.21

[a]Age and Gosner stage of tadpoles were not significant covariates and thus were removed from the final model.

increase in estimated species richness (Figure 2, Table 1). The difference in estimated species richness due to larval PCB-126 exposure was more pronounced in frogs, as shown by significant differences between control and PCB-treated frogs (Figure 2). Principal Coordinates Analysis plots exhibited little difference between the microbial community composition of control and PCB-treated tadpoles (Figure 3), and statistical analysis demonstrated that these groups were not significantly different (adonis: $p = 0.09$). However, there was obvious, differential clustering of frogs that underwent larval exposure to PCB-126 compared with the control frogs, and these results were statistically significant (adonis: $p = 0.03$; Figure 3).

DISCUSSION

We observed changes in the microbial community structure due to larval exposure to PCB-126. Although the present study only monitored changes in community structure, the results raise several questions and hypotheses that should be addressed to expand our understanding of the mutualisms between vertebrate hosts and gut microbes and the impact of anthropogenic pollutants on these relationships.

Control and PCB-treated tadpoles exhibited several significant differences in gut microbial diversity. Tadpoles exposed to PCB-126 lacked *Aminobacter*, a genus that was only hosted by control tadpoles [8]. These tadpoles also exhibited a higher prevalence of the genus *Pseudomonas*, some members of which are known to be pathogenic to amphibians [27]. Overall community diversity did not differ, however, between control and PCB-treated tadpoles, as compared using unweighted UniFrac distances and the adonis function.

Frogs exposed to PCB-126 during the larval stage exhibited several differences in microbial community structure. Frogs exposed to PCB-126 harbored a higher abundance of Fusobacteria. In addition, PCB-exposed frogs housed a more diverse microbial community in terms of estimated species richness. In total, the microbial community of PCB-treated frogs differed significantly from controls, as demonstrated by unweighted UniFrac distances and the use of the adonis function. These findings highlight the lasting effects of larval exposure to environmental pollutants on the anuran gut microbiota.

Dietary PCBs may interact with microbes directly to alter community structure, perhaps selecting for tolerant microbes. The addition of PCBs to soil tends to cause enrichment in community members that are capable of degrading PCBs [28]. Other xenobiotics, such as human pharmaceuticals, cause direct shifts in gut microbial communities [29]. In addition, frogs had accumulated PCB-126 in their tissues, which may have been excreted through bile into the gut, potentially interacting with the gut microbiota directly.

It is also likely that the effects of PCBs on the gut microbial community are mediated through host physiology. For example, we observed significantly higher estimated species richness in the PCB-treated animals compared with controls. These differences could be due to lowered immune function in PCB-exposed individuals, which has been demonstrated with other vertebrate taxa [10–12]. Altered gut immunity may allow
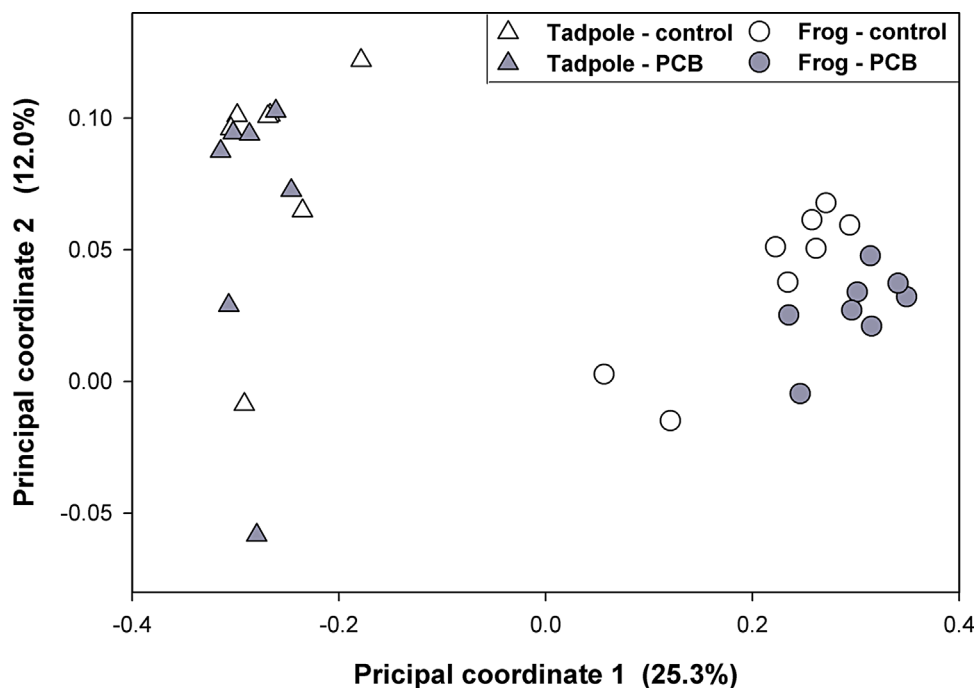

Figure 3. Principal Coordinate Analysis using unweighted UniFrac scores of the microbial communities from control and polychlorinated biphenyl-treated (PCB) tadpoles and frogs.

for opportunistic microbes to colonize the guts of toxin-exposed animals, thus increasing species richness and altering microbial community structure. For example, the genus *Pseudomonas*, a potential pathogen of amphibians [27], was only detected in PCB-treated tadpoles. Similarly, the genus *Fusobacterium* comprised a significant proportion of the microbial community of PCB-treated frogs, but not any other group studied. This genus contains numerous opportunistic pathogens that produce varied cytotoxic and necrotic virulence factors [30]. We cannot confirm the pathogenicity of the bacterial operational taxonomic units detected in the present study. Thus, the interactions between environmental pollutants, the immune system, opportunistic pathogens, and other members of the gut microbiota warrant further investigation.

Regardless of the mechanism, larval exposure to PCB-126 significantly altered the microbial community structure of the amphibian gut, which could affect host performance and fitness. The microbiota plays large roles in the energy balance [31], immune function [32], and even the behavior [33] of the host. Disrupting this community might impair the complex relationship between host and microbes, negatively affecting host fitness. In addition, if pollutants allow for increased colonization by opportunistic microbes, this effect may increase the probability of infection by pathogenic microbes. The field of ecotoxicology often focuses on how environmental pollutants impact various organs or physiological processes, and until recently, the impact of pollutants on symbiotic microbes has been largely overlooked. The present study highlights 2 areas of potential future research. First, the gut microbiota can vary between animals in both nature and captivity [34–36]; thus, it would be interesting to see whether differences similar to the present study are also found between pristine and contaminated sites in the wild. Furthermore, although we document several changes in microbial diversity and abundances of taxa, future studies could investigate the mechanisms that underlie these changes and the consequences for host health and fitness.

REFERENCES

1. Beebee TJC, Griffiths RA. 2005. The amphibian decline crisis: A watershed for conservation biology? *Biol Conserv* 125:271–285.
2. Coyle TLC, Karasov WH. 2010. Chronic, dietary polybrominated diphenyl ether exposure affects survival, growth, and development of *Rana pipiens* tadpoles. *Environ Toxicol Chem* 29:133–141.
3. Rosenshield ML, Jofré MB, Karasov WH. 1999. Effects of polychlorinated biphenyl 126 on green frog (*Rana clamitans*) and leopard frog (*Rana pipiens*) hatching success, development, and metamorphosis. *Environ Toxicol Chem* 18:2478–2486.
4. Christin MS, Ménard L, Gendron AD, Ruby S, Cyr D, Marcogliese DJ, Rollins-Smith L, Fournier M. 2004. Effects of agricultural pesticides on the immune system of *Xenopus laevis* and *Rana pipiens*. *Aquat Toxicol* 67:33–43.
5. Cary TL, Ortiz-Santaliestra ME, Karasov WH. 2014. Immunomodulation in post-metamorphic northern leopard frogs, *Lithobates pipiens*, following larval exposure to polybrominated diphenyl ether. *Environ Sci Technol* 10:5910–5919.
6. Van Schmidt ND, Cary TL, Ortiz-Santaliestra ME, Karasov WH. 2012. Effects of chronic polybrominated diphenyl ether exposure on gonadal development in the northern leopard frog, *Rana pipiens*. *Environ Toxicol Chem* 31:347–354.
7. O'Hara AM, Shanahan F. 2006. The gut flora as a forgotten organ. *EMBO Rep* 7:688–693.
8. Kohl KD, Cary TL, Karasov WH, Dearing MD. 2013. Restructuring of the amphibian gut microbiota through metamorphosis. *Environmental Microbiology Reports* 5:899–903.
9. Huang YW, Karasov WH, Patnode KA, Jefcoate CR. 1999. Exposure of northern leopard frogs in the Green Bay ecosystem to polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans is measured by direct chemistry but not hepatic ethoxyresorufin-O-deethylase activity. *Environ Toxicol Chem* 18:2123–2130.
10. Arkoosh MR, Clemons E, Myers M, Casillas E. 1994. Suppression of B-cell mediatedimmunity in juvenile chinook salmon (*Oncorhynchus tshawytscha*) after exposure to either a polycyclic aromatic hydrocarbon or to polychlorinated biphenyls. *Immunopharmacol Immunotoxicol* 16:293–314.
11. Finkelstein ME, Grasman KA, Croll DA, Tershy BR, Keitt BS, Jarman WM, Smith DR. 2007. Contaminant-associated alteration of immune function in black-footed albatross (*Phoebastria nigripes*), a North Pacific predator. *Environ Toxicol Chem* 26:1896–1903.
12. Sormo EG, Larsen HJS, Johansen GM, Skaare JU, Jenssen BM. 2009. Immunotoxicity of polychlorinated biphenyls (PCB) in free-ranging gray seal pups with special emphasis on dioxin-like congeners. *J Toxicol Environ Health Part A* 72:266–276.
13. Choi JJ, Eum SY, Rampersaud E, Daunert S, Abreu MT, Toborek M. 2013. Exercise attenuates PCB-induced changes in the mouse gut microbiome. *Environ Health Perspect* 121:725–730.
14. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
15. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* 6:1621–1624.
16. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Meth* 7:335–336.
17. Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461.
18. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes: A Chimera-checked 16 S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72:5069–5072.
19. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. 2009. PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26:266–267.
20. Price MN, Dehal PS, Arkin AP. 2009. FastTree: Computing large minimum-evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 26:1641–1650.
21. Wang Q, Garrity GM, Tiedja JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267.
22. Kumar PS, Mason MR, Brooker MR, O'Brien K. 2012. Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. *Journal of Clinical Periodontology* 39:425–433.
23. Shchipkova AY, Nagaraja HN, Kumar PS. 2010. Subgingival microbial profiles of smokers with periodontitis. *J Dent Res* 89:1247–1253.
24. Faith DP. 1992. Conservation evaluation and phylogenetic diversity. *Biol Conserv* 61:1–10.
25. Lozupone C, Knight R. 2005. UniFrac: A new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71:8228–8235.
26. Anderson MJ. 2001. A new method for non-parametric multivariate analysis of variance. *Australian Journal of Ecology* 26:32–46.
27. Brodkin MA, Simon MP, DeSantis AM, Boyer KJ. 1992. Response of *Rana pipiens* to graded doses of the bacterium *Pseudomonas aeruginosa*. *J Herpetol* 26:490–495.
28. Yan T, LaPara TM, Novak PJ. 2006. The reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl in three different sediment cultures: Evidence for the involvement of phylogenetically similar *Dehalococcoides*-like bacterial populations. *FEMS Microbiol Ecol* 55:248–261.

29. Maurice CF, Haiser HJ, Turnbaugh PJ. 2012. Xenobiotics shape the physiology and gene expreession of the active gut microbiome. *Cell* 152:39–50.

30. Nagaraja TG, Narayanan SK, Stewart GC, Chengappa MM. 2005. *Fusobacterium necrophorum* infections in animals: Pathogenesis and pathogenic mechanisms. *Anaerobe* 11:239–246.

31. Semova I, Carten JD, Stombaugh J, Mackey L, Knight R, Farber SA, Rawls JF. 2012. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host & Microbe* 12:277–288.

32. Round JL, Mazmanian SK. 2009. The gut microbiota shapes intestinal immune responses in health and disease. *Nat Rev Immunol* 9:313–323.

33. Heijtz RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. 2011. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci* 108:3047–3052.

34. Kohl KD, Skopec MM, Dearing MD. 2014. Captivity results in disparate loss of gut microbial diversity in closely related hosts. *Conservation Physiology* 2:cou009.

35. Villers LM, Jang SS, Lent CL, Lewin-Koh S-C, Norosoarinalvo JA. 2008. Survey and comparison of major intestinal flora in captive and wild ring-tailed lemur (*Lemur catta*) populations. *Am J Primatol* 70:175–184.

36. Wienemann T, Schmitt-Wagner D, Meuser K, Segelbacher G, Schink B, Brune A, Berthold P. 2011. The bacterial microbiota in the ceca of capercaillie (*Tetrao urogallus*) differs between wild and captive birds. *Syst Appl Microbiol* 34:542–551.