Received: 26 April 2022 | Accepted: 23 September 2022

DOI: 10.1111/1462-2920.16222

RESEARCH ARTICLE

environmental microbiology Applied Microbiology International

Xenobiotic estradiol-17ß alters gut microbiota of hatchling American alligators (*Alligator mississippiensis*)

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Funding information
Auburn University Department of Biological Sciences; Auburn University Museum of Natural History; IUCN-SSC Crocodile Specialist Group, Grant/Award Number: 19/3

Abstract

Environmental oestrogens pose serious concerns for ecosystems through their effects on organismal survival and physiology. The gut microbiome is highly vulnerable to environmental influence, yet the effects of oestrogens on gut homeostasis are unknown because they are poorly studied in wildlife populations. To determine the influence of environmental oestrogens (i.e., xenoestrogens) on the diversity and abundance of gut microbiota, we randomly assigned 23 hatchling American alligators (*Alligator mississippiensis*) to three ecologically relevant treatments (control, low, and high oestrogen concentrations) for 10 weeks. We predicted that xenoestrogen exposure would decrease microbial diversity and abundance within the digestive tract and that this effect would be dose-dependent. Microbial samples were collected following diet treatments and microbial diversity was determined using 16S rRNA gene-sequencing. Individuals in oestrogen-treatment groups had decreased microbial diversity, but a greater relative abundance of operational taxonomic units than those in the control group. In addition, this effect was dose-dependent; as individuals were exposed to more oestrogen, their microbiome became less diverse, less rich and less even. Findings from this study suggest that oestrogen contamination can influence wildlife populations at the internal microbial-level, which may lead to future deleterious health effects.

INTRODUCTION

The environment plays an integral role in shaping patterns of development, survival and reproduction (Deeming et al., 1991; Gilbert & Epel, 2015; West-Eberhard, 2003), but contaminants can negatively affect these components of fitness through their influence on important physiological processes. Endocrine-disrupting contaminants (EDCs), for example, can enter the environment through agricultural run-off and spills, disrupting organismal processes such as endocrine physiology (e.g., gene expression; Kazeto et al., 2004) and gonadal development (Guillette Jr et al., 1994; Jobling et al., 1998; Sparling, 2016). Synergism of multiple EDCs in ecosystems can also exacerbate the complexity of effects on physiology (Swan et al., 2005). Consequently, these compounds can have downstream effects that can harm individual and population health (Finger & Gogal, 2013; Milnes & Guillette Jr, 2008).

EDCs include compounds such as pesticides, fungicides and pharmaceuticals (Finger & Gogal, 2013; Guillette Jr et al., 2007; Guillette Jr & Iguchi, 2003; Sparling, 2016), many of which are biochemically similar to oestrogen and arise from a range of natural and synthetic sources. Since oestrogen receptors are present throughout the body (Deroo & Korach, 2006), exposure to xenoestrogens can disrupt synthesis, transport, storage and clearance of hormones (Guillette Jr et al., 2007; Milnes & Guillette Jr, 2008; Sparling, 2016). Estradiol-17ß (E2) is one of the most active oestrogens and plays a key role in regulating growth and reproductive function (Fernandez & Russo, 2010; Simpson, 2003; Yang et al., 2017).

Because E2 does not readily degrade under anaerobic conditions (half-life of 2–3 days in river water; Ying et al., 2002; Ying et al., 2003), it can remain active in certain environments and is often detectable in treatment plant effluents and surface water (Adeel et al., 2017; Desbrow et al., 1998; Kuch & Ballschmiter, 2001; Ternes et al., 1999; Ying et al., 2002). Indeed, xenobiotic exposure to E2 has been shown to influence animal health and physiology in aquatic systems (Clark et al., 1998; Irwin et al., 2001; Miles-Richardson et al., 1999; Palmer & Palmer, 1995; Toft & Baatrup, 2001). Exposure to concentrated amounts of E2 can occur directly from the environment or through the consumption of bioaccumulated prey items and influence bodily function at the cellular level. In fact, gut microbial activity can drive oestrogen metabolism (Adlercreutz & Järvenpää, 1982; Eriksson, 1970; Plottel & Blaser, 2011).

The gut microbiome modulates oestrogen levels within the body by secreting ß-glucuronidase (GUS) enzymes, which enable oestrogen to become biologically active (Baker et al., 2017; Ervin et al., 2019; Plottel & Blaser, 2011). In addition, the presence of oestrogen receptors in the epithelia of the gastrointestinal (GI) tract (Yang et al., 2017) further indicates that the gut may be an important site for oestrogen interactions. The 'estrobolome', or the gene repertoire of gut microbiota that metabolize oestrogens, can influence the levels of circulating and excreted oestrogens, as well as oestrogen metabolites (Plottel & Blaser, 2011). The liver is responsible for conjugating E2 to its inactive form (estradiol-17-glucuronide). However, GUS enzymes secreted by bacteria in the GI tract deconjugate E2, which allows oestrogen in its biologically available form to recirculate through the bloodstream and possibly contribute to hormonal disorders and diseases at high levels (Ervin et al., 2019).

An estrobolome enriched in GUS-producing genes can promote oestrogen metabolite deconjugation, leading to more unbound oestrogens in the plasma and bodily tissues to potentially cause damage (Ervin et al., 2019). In addition, ethinyl estradiol (a synthetic oestrogen) can alter intestinal flora in a sex-dependent and transgenerational manner in rodents by increasing the proportions of potentially pathogenic bacteria (Javurek et al., 2016). Estradiol has been shown to decrease gut microbiota diversity in zebrafish (Liu et al., 2016), and mediate phenotypic effects on zebrafish through the presence or absence of microbiota (Catron et al., 2019). Indeed, this imbalance of gut microbiota in individuals exposed to contaminants is not uncommon (Aguilera et al., 2020; Evariste et al., 2019). A symptomatic effect of contaminant exposure, either through direct facilitation of a toxin in the gut or by a cascade of physiological responses that influence microbiota composition, is a decrease in microbial diversity (Aguilera et al., 2020). This decrease in diversity, including compositional and functional diversity, is linked to a variety of deleterious health effects (Carding et al., 2015). However, the direct influence and consequences of xenoestrogens on gut microbial diversity is largely unknown.

American alligators (*Alligator mississippiensis*; Order Crocodilia) are an effective model to study environmental health and the effects of EDCs (Finger & Gogal, 2013; Tavalieri et al., 2020). *Alligator mississippiensis* is a long-lived, top-trophic predator, meaning that EDC concentrations and physiological effects may be biomagnified (or have increased deposition) in relatively old individuals (Heinz et al., 1991; Roe et al., 2004; Finger & Gogal, 2013; Tavalieri et al., 2020). In addition, like other reptiles, environmental conditions mediate development (Joanen & Mcnease, 1989), growth (Lance, 2003), and homeostasis (Seebacher et al., 2003) in *A. mississippiensis*. These characteristics make alligators an excellent model to address whether EDCs, including environmental E2, might influence GI microbiota. Past studies have identified significant microbial variation within the GI tract of alligators (Keenan et al., 2013) and the influence of heavy metals on microbiota composition (Kieran et al., 2020); however, nothing is known regarding the physiological influence of xenoestrogens on the alligator gut microbiome.

The aim of this study is to investigate whether xenobiotic oestrogens, specifically E2, can influence the diversity and abundance of gut microbiota in hatchling alligators. Given the decreased microbial diversity following E2 exposure documented in other studies, we predict that (1) xenoestrogen exposure will decrease microbial diversity and abundance within the digestive tract and that (2) the effect of xenoestrogens on the gut microbiome will be dose-dependent, whereby higher concentrations will have a greater impact than lower concentrations. Experimental tests of these predictions will provide a novel assessment of how oestrogens can influence important microbiota in wildlife populations.

EXPERIMENTAL PROCEDURES

Transport and housing

Twenty-three hatchling (<1 year old) *A. mississippiensis* were obtained from Rockefeller Wildlife Refuge (RWR; Grand Chenier, LA, USA) and transported to Auburn University's Aviary Facility (Auburn, AL) on 4 March 2019. Eggs were collected from natural nests at RWR and incubated under lab conditions at an average of 30.6°C. Upon hatching, individuals were communally housed in controlled environmental chambers. Alligators were transported via containers drilled with holes to allow for air circulation and were blindfolded to potentially reduce stress

(Finger, Hamilton, et al., 2018; Finger, Kelley, et al., 2018). Upon arrival to Auburn University, alligators were individually housed in 23 clear, plastic tanks (30 cm × 44 cm × 66 cm). Each tank was equipped with an individual UV light and one wooden basking platform and filled with approximately 17–18 L of water. Photoperiod was kept at a 12–12 h light–dark cycle and air temperature was maintained above 25°C. Water temperature was documented twice a day via two digital temperature probes randomly placed in six tanks. Water in tanks was replaced once per week a day after feeding.

Prior to E2 exposure, alligators were housed for 10 weeks to allow for dietary transition (Kohl et al., 2017). During this time, individuals were fed thawed day-old pinky mice (weighing 1–2 g; The Big Cheese Rodent Factory, Fort Worth, TX, USA) twice a week. Mice were thawed for 15 min prior to feeding and were then placed into tanks. If not eaten, mice were removed the following day during cleaning.

Individuals were weighed once per week following arrival and throughout the treatment period. In addition, 6–8-month-old crocodilians can eat about 4% of their body weight (in grams) per week under captive conditions (Webb et al., 1991). Thus, we fed alligators in our study ~5% of their body weight per week to eliminate potential effects of under- or over-feeding. Importantly, digestive efficiency may be low when the stomach is repeatedly filled to capacity, as observed under captive conditions (Webb et al., 1991).

Experimental design and sample collection

On 31 May 2019, individuals were randomly assigned to three treatments. These treatments included individuals with no E2 exposure (control treatment, $n = 7$), individuals that received 0.5 μg/kg E2 (low dose treatment, $n = 8$), and individuals that received 1 μg/kg E2 (high dose treatment, $n = 8$). Alligators were visually sexed prior to treatment assignment and similar distributions of males and females were placed into the groups. Sex was confirmed during dissection after the experiment concluded. Dosage ranges were chosen to mimic ecologically relevant concentrations of E2 at polluted sites [351–957 ng/L (approximately 0.957 μg/kg) in dairy farm wastewater and 1000–1500 ng/L in swine farm effluents (approximately 1.5 μg/kg; Adeel et al., 2017; Li Yx & Lin, 2010)]. These concentrated animal feeding operations have been shown to influence the physiology of aquatic wildlife through pollution of wastewater that drains into natural ponds and streams (Orlando et al., 2004).

Estradiol-17ß (Sigma Aldrich) was prepared by dissolving the powdered form into peanut oil and diluting to desired concentrations. Mice were injected with 0.5 μg/kg, 1 μg/kg, or a peanut oil control at a volume of 1 ml/kg using a 1 ml syringe. Any associated oestrogen in pinky mice was considered to not influence the results of this study as oestrogen concentration in foetal mice is very low (Saal et al., 1997) and would be equal across treatments. Alligators were blindfolded and then fed by oral gavage to ensure all dosages of E2 were consumed. This involved inserting pinky mice directly into the oesophagus and massaging the throat gently with their mouth held closed, a technique used in previous studies (Finger, Hamilton, et al., 2018). Individuals were closely monitored following feeding and regurgitation was not observed. Alligators were orally gavaged with two pinky mice injected with E2 each week; thus, 20 total doses of a control or E2 were given to hatchlings.

Following the 10-week dosage period, individuals were weighed to the nearest 0.1 g and euthanized via decapitation and subsequent cervical dislocation and pithing (Nevarez et al., 2014). Twenty millilitres of heparinized blood samples were collected following decapitation and centrifuged; plasma was extracted to analyse blood E2 concentrations. Microbial samples were aseptically excised along the digestive tract using sterile scalpel blades and immediately frozen at −80°C. Samples were collected along three sections of the gastrointestinal (GI) tract including stomach, duodenum, and colon (Keenan et al., 2013). Samples from the duodenum were prioritized for sequencing and further analyses because of this region's important role in nutrient absorption. In addition, destructive sampling was selected over faecal sampling due to the lack of GI tract microbiota representation in the faecal microbiome of *A. mississippiensis* (Keenan et al., 2013).

The day following sample collection, total nucleic acids were extracted using a Zymo Research *Quick*-DNA Faecal/Soil Microbe Miniprep kit, following the manufacturer's instructions. Nucleic acid yield was quantified using Nanodrop and stored at −80°C prior to 16S rRNA gene-sequencing.

Sample analysis and microbial inventories

Heparinized blood samples were collected during dissections and all blood samples were stored on ice until they were centrifuged approximately 1–4 h following collection; plasma was then stored at −80°C. Hormonal steroids were thawed and extracted using 3 ml of diethyl ether and dried down with nitrogen gas. Plasma was then resuspended in a phosphate buffer solution and aliquoted in duplicate (200 μl each) into the respective tritiated hormone and antibody. Plasma oestrogen levels were quantified using two ELISA estradiol kits (Caymen Chemical) following the manufacturer's instructions. Extraction and radioimmunoassay procedures followed previously established protocols (Mendonça et al., 1996). Values were corrected for

volume of plasma and are reported in picograms per millilitre of plasma.

To determine the role of treatment on levels of E2 in the bloodstream, plasma estradiol concentrations from individuals were analysed using a linear model with treatment as an independent variable and alligator mass and sex as covariates. If interactions were not significant, they were excluded from the model. In addition, effects of E2 exposure were analysed using a generalized linear model with alpha diversity metrics (see below) as dependent variables with mass and sex included as covariates. Because all hatchlings were hatched and housed together at RWR, clutch ID was unknown and thus, not incorporated into statistical models. All statistical analyses were performed using RStudio software version 1.0.153 (R Development Core Team 2018) with the package *lme4* (Bates et al., 2007), and resulting figures were created utilizing colour-blind friendly palettes. Raw means ± *SE* are reported for treatment groups where linear model regression coefficients are β.

Microbial samples were sent to the University of Georgia for 16S rRNA gene-sequencing in triplicate. Gene libraries for the 16S rRNA gene were prepared by the 2-step Adapterama Quadruple-index PCR method (Glenn et al., 2019) using the primers S-DBact-0341-b-S-17 (Bakt_341F) CCTACGGGNGGCWGCAG and S-DBact-0785-a-A-21 (Bakt_805R) GACTACHVGGGTATCTAATCC (Klindworth et al., 2013). This method resulted in 46 base-pair reads. Sequences were demultiplexed via Mr. Demuxy software (https://pypi.org/project/Mr_Demuxy/1.2.0/; Glenn et al., 2019) and imported into QIIME2 version 2020.2 (Bolyen et al., 2019; Caporaso et al., 2010) where they were denoised and chimeras removed using DADA2 (Callahan et al., 2016). Feature tables were filtered and classified into operational taxonomic units (OTUs) using sklearn on a pre-trained 99 OTU GreenGenes v 13.8 database (DeSantis et al., 2006). Any OTUs identified in the negative control sample were considered contaminants and thus removed from the following analyses (Salter et al., 2014); however, contamination was not present in any of the sequence files. Unassigned OTUs, which made up 0.005% of total sequences, were also removed from the dataset. Because samples were sequenced in triplicate, we used the average of OTU absolute abundances in all three files for downstream analyses. Sequences and datasets were used to calculate rarefaction curves which revealed high to moderate sample coverage (Figure S1).

Alpha diversity was identified using several measures: OTU absolute abundance, Shannon's diversity index, and Pielou's evenness index (Pielou, 1966). OTUs were grouped according to genus and phylum and analysed using a linear fixed effects model with OTU as a dependent variable and treatment group as an independent variable. Mass and sex were also included as covariates in the analyses. If interactions were not significant, they were removed from the models. Other alpha diversity metrics (e.g., phyla and genera relative abundances) were analysed using linear fixed effects models as well, however, sex and mass were not included as covariates. Beta diversity was compared using a non-metric multi-dimensional scaling analysis (NMDS) to calculate the level of similarity between bacterial community composition using the Bray–Curtis dissimilarity index in the R *vegan* package (Oksanen et al., 2007), where each point in the plot is an individual sample. This metric was quantified using an analysis of similarity (ANOSIM, permutations = 999). All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under Accession Number PRJNA831851. All scripts and datasets have been uploaded to GitHub (https://github.com/kmm0155/Alligator-Gut-Microbiome-Murphyetal). The main dataset used in statistical models can be found as Table S1.

RESULTS

Plasma oestrogen

Individuals in the high treatment group had an average of 143 pg/ml ($SE = 51.05$) more E2 in their plasma than control individuals ($p = 0.01$; Figure 1). No significant difference was detected between low dose and control groups ($\beta = 41.08$, $SE = 51.48$, $p = 0.44$), nor between low and high dose groups ($\beta = 102.06$, $SE = 51.42$, $p = 0.06$), but there was a trend for the high dose individuals to have a higher concentration of E2 than those in the low treatment. In addition, females had 163 pg/ml more E2 in their plasma than males across treatment groups ($SE = 43.29$, $p = 0.001$). Mass was also significantly correlated with plasma E2 levels ($\beta = 2.16$, $SE = 0.85$, $p = 0.02$; Figure S2).

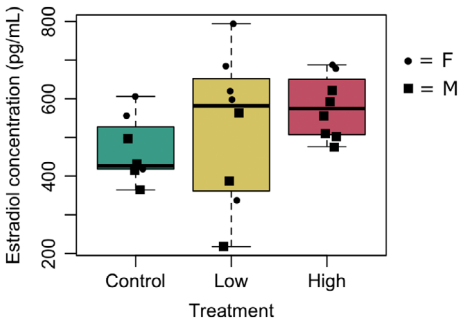

FIGURE 1 Estradiol-17ß (E2) concentrations in plasma of hatchling *Alligator mississippiensis* exposed to no E2 (Control) and two concentrations of E2 (low and high) are represented as boxes, while the whiskers extending from the boxes represent the 1st and 3rd quartiles ±1.5× of the interquartile range. The bold line represents median values within the box. Females are represented as circles and males are represented as squares.

Microbial sequences

A total of 10,899 OTUs were utilized for analyses following blank sample and unassigned OTU removal. Individuals in the low-dose treatment group had 133 more bacterial OTUs within the GI tract than control treatment individuals ($SE = 61.78$, $p = 0.04$; Table 1). However, high treatment individuals did not differ in OTU absolute abundance from control treatment individuals ($\beta = 91.82$, $SE = 61.30$, $p = 0.15$; Figure 2A). No significant difference in number of OTUs were detected between high and low dose treatments ($\beta = 41.01$, $SE = 60.72$, $p = 0.51$). In addition, OTU absolute abundances were not correlated with plasma E2 values ($\beta = 0.26$, $SE = 0.25$, $p = 0.31$) or associated with alligator body mass ($\beta = 0.39$, $SE = 1.20$, $p = 0.74$) and sex ($\beta = 45.96$, $SE = 61.97$, $p = 0.47$). However, the generalized linear model revealed a correlation between E2 exposure and OTU absolute abundance where xenobiotic exposure to E2 increased aabundances ($\beta = 112$, $SE = 51.6$, $p = 0.043$; Table 2).

TABLE 1 Effect of estradiol-17β (E2) treatment on alpha diversity metrics in the gut microbiome and E2 concentrations in plasma of hatchling *Alligator mississippiensis*.

	Control	Low treatment		High treatment		Overall model values
Dependent variable	**Mean**	**Mean**	**$\beta \pm SE$**	**Mean**	**$\beta \pm SE$**	***F*-value, *DF*, *p*-value**
OTU absolute abundance	400	537	133 ± 61.78	476	92 ± 61.27	2.10, 20, 0.12
Proteobacteria	134	243	109.5 ± 54.3	299	164.8 ± 54.3	4.72, 20, **0.021**
Helicobacter sp.	134	240	106.3 ± 53.6	297	163.3 ± 53.6	4.74, 20, **0.021**
Shannon's diversity index	2.74	2.606	−0.178 ± 0.22	2	−0.63 ± 0.22	3.12, 20, **0.035**
Pielou's evenness index	0.751	0.694	−0.047 ± 0.05	0.611	−0.15 ± 0.05	4.01, 20, **0.009**
Blood plasma E2 (pg/ml)	470	525	41.08 ± 51.5	578	143 ± 51.05	5.97, 20, **0.003**

Note: Sex and mass were included as covariates in the linear fixed effects model. Effect sizes (β) utilize the control as a reference group. Statistically significant *p* values are in bold face. OTU = operational taxonomic unit.

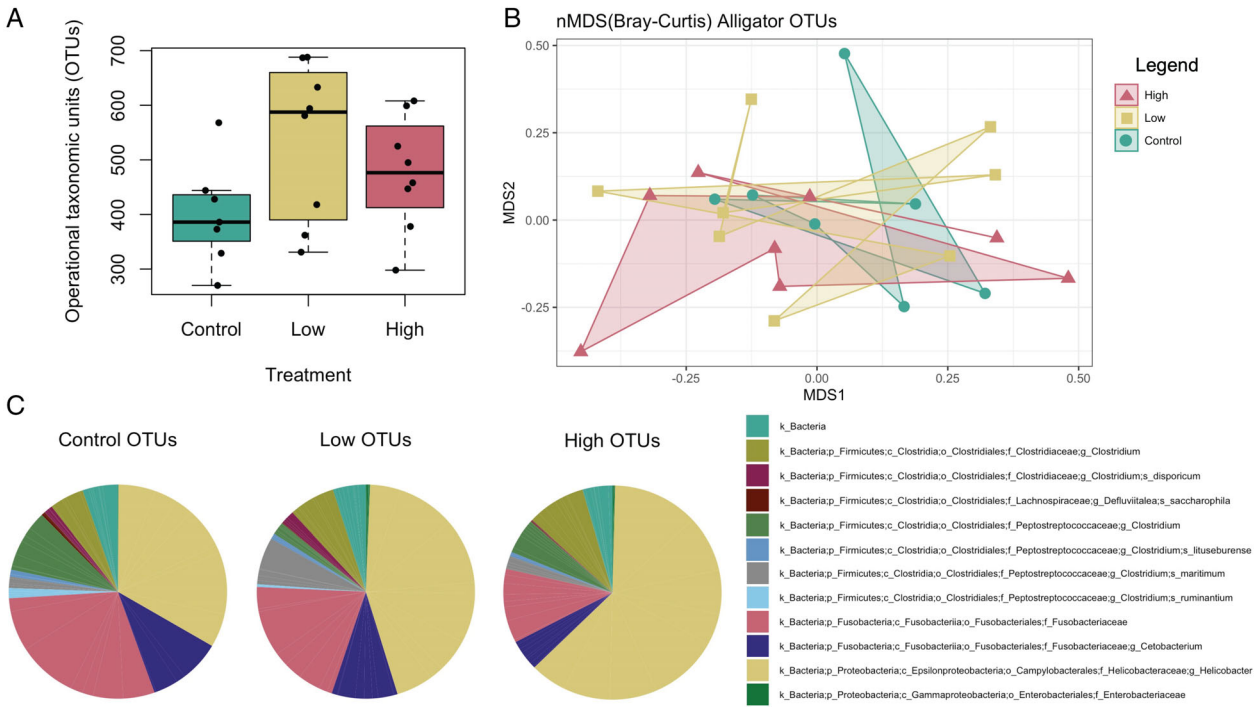

FIGURE 2 Variation in microbial communities and abundance across three treatment groups (i.e., level of exposure to estradiol-17ß) in hatchling *Alligator mississippiensis*. (A) Differences in microbial operational taxonomic unit (OTU) absolute abundance among treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ±1.5× interquartile range, while the bold line represents median values within the box. (B) Non-metric multidimensional scaling (NMDS) plot of all individuals. The green circles in the NMDS plot are for individuals in the control treatment, yellow squares are for individuals in the low estradiol treatment, and red triangles are for individuals in the high estradiol treatment. Lines denote vector overlays on NMDS ordination, indicating the directionality and strength of change (line length) for a specific parameter. (C) Differences in bacterial OTU relative abundances among treatment groups (i.e., control, low, and high) where kingdom (k), phylum (P), class (c), order (O), family (F), genus (G), and (s) are indicated in the figure legend.

TABLE 2 Effect of sex, mass and estradiol-17β (E2) treatment using a generalized linear fixed effects model on alpha diversity in the gut microbiome of hatchling *Alligator mississippiensis*

	Sex effect		Mass effect		E2 treatment effect	
Dependent variable	$\beta \pm SE$	***p*-value**	$\beta \pm SE$	***p*-value**	$\beta \pm SE$	***p*-value**
OTU absolute abundance	-81.2 ± 47.9	0.11	1.14 ± 0.99	0.26	112 ± 52	**0.043**
Shannon's diversity index	-2.35 ± 1.07	**0.04**	-0.01 ± 0.006	**0.03**	-0.41 ± 0.21	0.06
Pielou's evenness index	0.03 ± 0.04	0.54	-0.0003 ± 0.001	0.77	-0.01 ± 0.05	0.052

Note: Effect sizes are represented as 'β'. Statistically significant *p*-values are in bold face.
Abbreviation: OTU, operational taxonomic unit.

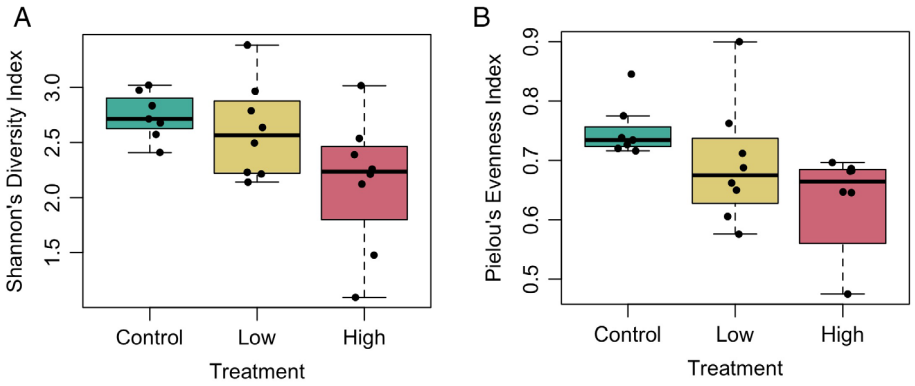

FIGURE 3 Variation in Shannon's diversity and Pielou's evenness indices across three treatment groups (i.e., level of exposure to estradiol-17β) in hatchling *Alligator mississippiensis*. (A) Shannon's diversity index scores and (B) Pielou's evenness index scores for each treatment group are represented as boxes, while the whiskers extending from the boxes represent the 1st and 3rd quartiles $\pm 1.5\times$ of the interquartile range. The bold line represents median values within the box.

Three bacterial phyla were detected across the 23 individual alligators: Proteobacteria (51% of absolute phyla across treatment groups), Fusobacteria (29% of absolute phyla across treatment groups), and Firmicutes (20% of absolute phyla across treatment groups). The high treatment group had a significantly greater relative abundance of Proteobacteria than the control treatment group ($\beta = 164.8$, $SE = 54.27$, $p = 0.007$; Table 1). In addition, the low treatment group had more Proteobacteria than the control group ($\beta = 109.5$, $SE = 54.27$, $p = 0.057$; Table 1). The high and low treatment groups did not differ in terms of relative abundance of Proteobacteria ($p = 0.305$).

There were no bacterial groups that were present in one treatment group and absent in another. Bray–Curtis NMDS showed different patterns of clustering of samples across treatment groups (Figure 2B). However, bacterial community structure did not differ between groups ($R = 0.026$, $p = 0.28$), suggesting that microbiomes of each treatment group were similar.

Four bacterial genera were detected across treatment groups: *Helicobacter* (64% of absolute genera across treatment groups), *Clostridium* (25% of absolute genera across treatment groups), *Cetobacterium* (11% of absolute genera across treatment groups), *Defluviitalea* (0.03% of absolute genera across treatment groups). Again, the high treatment group had a significantly greater relative abundance of *Helicobacter*, a Proteobacterium, than the control treatment group ($\beta = 163.3$, $SE = 53.57$, $p = 0.006$; Figure 2C).

Based on Shannon's diversity index, the high treatment group was less rich and less even than the control treatment ($\beta = 0.63$, $SE = 0.22$, $p = 0.01$; Table 1 and Figure 3A). However, high treatment individuals had a greater Shannon's index than low treatment individuals ($\beta = 0.45$, $SE = 0.22$, $p = 0.06$; Table 1 and Figure 3A). Mass had a significant effect on Shannon's index, whereby heavier individuals had a lower Shannon's index than those that weighed less ($\beta = 0.012$, $SE = 0.005$, $p = 0.041$). In addition, a significant interaction was detected between body mass and sex when comparing Shannon's index ($\beta = 0.016$, $SE = 0.007$, $p = 0.048$), whereby Shannon's index was negatively correlated with body mass; however, this effect is not seen as strongly in males. Plasma E2 values were also negatively correlated with Shannon's index ($\beta = 0.002$, $SE = 0.0009$, $p = 0.014$). Analyses of Pielou's index revealed similar results to those for Shannon's diversity index. The high treatment group was less rich and diverse than the control group ($\beta = 0.15$, $SE = 0.05$, $p = 0.009$; Table 1 and Figure 3B), but no differences were detected between low treatment and control groups ($\beta = 0.05$, $SE = 0.05$, $p = 0.38$) nor between low and high treatment groups ($\beta = 0.10$, $SE = 0.05$,

$p = 0.06$). Plasma E2 levels were also negatively correlated with Pielou's index ($\beta = 0.0005$, $SE = 0.0002$, $p = 0.013$). In addition, mass ($\beta = 0.001$, $SE = 0.001$, $p = 0.28$) and sex ($\beta = 0.05$, $SE = 0.05$, $p = 0.36$) did not influence Pielou's index.

DISCUSSION

Concentrations of EDCs, including environmental oestrogens such as E2, in aquatic ecosystems are major determinants of population health for a variety of species and potentially have strong ecosystem-wide effects. Physiology at various life-history stages can be influenced by xenoestrogen pollutants and in turn, can have important consequences on reproduction. E2 has been shown to influence gut homeostasis (Liu et al., 2016). However, these associations are not well understood, particularly in wildlife populations. In this study, we administered E2 to hatchling alligators and demonstrated that xenoestrogen treatment decreased microbial diversity in the GI tract. However, this pattern does not support part of our first prediction because E2 increased gut bacterial absolute and relative abundances, particularly in specific groups of Proteobacteria. Interestingly, the low treatment group had more OTUs than either the control or high treatment groups. This effect was also dose-dependent, whereby increased exposure to E2 reduced the diversity, richness, and evenness the gut microbiome.

Blood plasma analysis revealed that treatment individuals had higher E2 concentrations than control individuals. Notably, females had greater E2 blood plasma levels than males and mass was positively correlated with E2 values. These findings are similar to previous studies in *A. mississippiensis*, where EDC exposure elevated E2 blood plasma concentrations that were also associated with both sex and size (Milnes et al., 2002). These data suggest that xenobiotic E2 may (1) increase blood plasma E2 regardless of sex, (2) increase blood plasma E2 in females at less than a year of age, and/or (3) increase overall mass of individuals following short-term (i.e., 10 weeks) exposure.

Similar to previous studies in zebrafish and rodent models (Javurek et al., 2016; Liu et al., 2016), E2 treatment affected gut bacterial diversity in alligators. For example, experimental exposure of male zebrafish to E2 and bisphenol A (BPA) reduced Shannon's diversity index in comparison to untreated males (Liu et al., 2016). Moreover, this work demonstrated a dominating effect of bacterial phylum CKC4 along with decreased diversity (Liu et al., 2016). Likewise, we saw an increase in one particular bacterial phylum (i.e., Proteobacteria) in low- and high-treatment individuals (Figure 2). This decrease in bacterial diversity could be attributed to the interspecies competition of bacteria in the GI tract and/or E2 decreasing nutrient availability, amount and so on, whereby some species may no longer thrive (Baker et al., 2017). In addition, our study revealed a negative correlation between mass and Shannon's diversity index. Heavier individuals seemed to have less rich and even microbial gut communities than their lighter counterparts. However, this finding did not remain when analysing Pielou's evenness index, suggesting that the relationship between mass and microbial diversity may depend on which diversity index is used by researchers.

The bacterial taxon that dominated the GI tract of individuals in the high treatment group was a Proteobacteria genus, *Helicobacter*. This often pathogenic genus is opportunistic in that following a disruption in homeostatic bacterial GI communities, *Helicobacter* can thrive and dominate intestinal environments. This increase in abundance, or the imbalance of *Helicobacter* with other bacterial taxa, contributes to a disrupted microbiome. Changes in microbiota community composition have been shown to cause inflammatory responses and exhibit metabolic profiles that can negatively influence gut epithelial health (Baker et al., 2017; Turnbaugh et al., 2006). *Helicobacter pylori* is responsible for chronic gastritis and peptic ulcers and is considered a pathogenic bacteria that is linked to gastric cancer in humans (Graham, 1991; Lofgren et al., 2011; Ohtani et al., 2007). Surprisingly, other studies have shown that E2 acts bateriostatically on *H. pylori* in vitro (Hosoda et al., 2011) and that *H. pylori* can absorb and hold E2 within its membrane (Hosoda et al., 2009). All *Helicobacter* species are associated with vertebrate hosts, show high host-specificity, and are mostly associated with the gastric, enteric, and hepatobiliary tracts (Gilbert et al., 2014, 2019).

Given the potential interaction between E2 and *Helicobacter*, and that E2 treatment increased *Helicobacter* abundance in our study, we suggest two main reasons for this observed change. First, Proteobacteria increase is often an indicator of disease or illness (Rizzatti et al., 2017). Indeed, treatment with pesticides can increase pathogenic bacteria (including *Helicobacter*) levels in rats (Yang et al., 2019). Other agricultural pollutants, such as glyphosate-based herbicides, decrease potentially beneficial microbes in young Japanese quail (Ruuskanen et al., 2020). E2 treatment in our study increased Proteobacteria species like *Helicobacter*, which suggests an imbalanced gut microbiota profile. Second, as carnivores and carrion-eaters, crocodilians are known to carry pathogenic bacteria within their GI tracts (Keenan et al., 2013; Lin et al., 2019; Willson et al., 2019). The abundances of these internal pathogenic bacteria, like *Helicobacter*, might assist with "inoculating" individuals against pathogens consumed through carrion and any bacterial toxins produced (Roggenbuck et al., 2014). We also identified *Clostridium* sp. in our study which has been associated with pathogenesis. A recent study by Kieran

et al. (2020) shows increases in *Clostridium* due to Selenium (Se) exposure in sexually immature *A. mississippiensis* following their seven-week experiment. Similar to E2, Se is necessary for endocrine function, but can induce toxicity at high levels (Janz et al., 2010).

One of the most interesting results from our study shows that exposure to a relatively low concentration of E2 increases microbiota absolute abundances but does not influence microbiota diversity. This dose-dependent effect suggests that varying concentrations of E2 may influence microbial community composition, in terms of alpha and beta diversity, in different ways. We suggest two reasons for why this may be: (1) Perhaps there is a three-way interaction occurring between genetics of individuals, their associated microbiota, and bodily concentrations of E2. Previous studies illustrate that xenoestrogens can affect alligator physiology (Guillette Jr et al., 1994; Guillette Jr et al., 2007), and that these physiological effects can influence gut microbiota community structure or vice versa. We suggest, however, that there may be reciprocal effects between the concentration (and potentially exposure time) of E2 and gut microbiota where gut microbiota may influence E2 concentrations in wildlife. Indeed, microbiota can metabolize oestrogens to their biologically-active form (Baker et al., 2017; Ervin et al., 2019; Plottel & Blaser, 2011). It has been suggested that microbiota can thereby influence the circulating levels of oestrogen in the body (Plottel & Blaser, 2011); however, no study thus far has demonstrated that altering gut microbiota patterns can change E2 plasma concentrations over time. In addition, the term 'endobolome' has been used to define microbial genes and their pathways that are involved in metabolism of steroid hormones and EDCs (Aguilera et al., 2020). Because our study did not analyse enhanced gene pathways or metabolites produced by the gut microbiota, we cannot conclude whether this change in community diversity altered circulating E2 levels through these mechanisms. (2) Perhaps genetic contributions to variation in the gut microbiome are reduced at a high concentration of E2, and genetic influences are only observed when exposed to E2 at low concentrations. Such interactive effects between genotype and E2 exposure could have different influences on abundance and diversity of gut microbiota. Indeed, this bi-phasic effect of EDCs on physiology is not unknown, and is attributed to the up- or down-regulation of genes by EDC compounds and to the response to natural hormones in the body (leading to the production of more or less hormone; Vandenberg et al., 2012). Previous studies have also demonstrated that following exposure to E2 alone, *A. mississippiensis* develops the same reproductive abnormalities observed in natural populations subject to complex mixtures of EDCs (Hale & Parrott, 2020). Thus, synergism of multiple EDCs in ecosystems may influence the gut microbiota in ways that E2 alone would. This idea may be addressed in future studies and would contribute to our knowledge of the complex interactions between gut microbiota, EDCs, and population health.

A limitation of our study is that we cannot identify whether the gut microbiome is modulated directly by E2 itself versus an E2-induced physiological response by an individual alligator. Environmental chemicals can induce changes in the gut microbiome, although many of these regulating mechanisms remain unknown (Chiu et al., 2020). For example, carbendazim, a widely-used fungicide, induces a change in gut microbiota community composition by increasing lipid absorption in the intestine which can cause multi-tissue inflammatory responses (Jin et al., 2018). If environmental E2 is contributing to the already circulating pool of oestrogen in the body, this influx could overwhelm the liver (whose role is conjugating unbound E2 to its inactive form, estradiol-17-glucuronide) and the oestrogen that the liver can conjugate is then unbound by GUS enzymes in the GI tract. Thus, individuals would have higher blood E2 concentrations, which we also found in our study between high treatment group individuals and the controls. This would contribute to the idea that it may be the body's physiological response, mediated by transcriptional factors, to an increase in circulating E2 rather than the interaction between GUS enzymes and bacterial growth. In addition, we prioritized a region of the digestive tract that is not as rich and diverse in microbiota as compared to the lower GI tract in alligators (Keenan et al., 2013). If samples taken from the colon of alligators used in this study had been sequenced, we might have seen even greater differences across treatment groups. It should be noted that the alligators' young age may have contributed to fewer OTUs present than those shown in other studies (Keenan et al., 2013; Kieran et al., 2020). Indeed, the number of OTUs present in human GI tracts is low at birth and bacterial diversity increases with age across populations (Yatsunenko et al., 2012). In addition, the gut microbiome is thought to develop through environmental exposure in reptiles (Colston & Jackson, 2016).

Captive housing conditions may have lowered the diversity of bacterial communities across time as well. Captive housing significantly alters bacterial diversity in lizards (Kohl et al., 2017), and the same may apply for alligators; however, farm-raised alligators have similar bacterial community structure compared to their wild counterparts (Keenan et al., 2013). In addition, during the acclimation period prior to xenobiotic E2 exposure, individuals were not subject to force-feeding. During the treatment period, hatchlings were fed by oral gavage. This difference in feeding technique may attribute some variation in gut microbiota communities where previous studies have shown that exposure to acute stress may alter the composition, function, and metabolic activity of gut microbiota (Karl et al., 2018).

However, because control individuals were also subject to this change in feeding technique, potential variation due to stress should be accounted for. Lastly, because individuals were hatched and housed under communal conditions at RWR prior to transportation to Auburn University, clutch ID was unknown and could not be incorporated as a random effect in statistical models. Perhaps variation in our results could be attributed to *in ovo* bacterial communities acquired from maternal sources that may have influenced the colonization and development of hatchling gut microbiota (Trevelline et al., 2018). Future studies should incorporate these factors into the experimental design and statistical analyses.

Studies of captive wildlife can provide important insights into the functional roles of microbiota in disease and health (Williams et al., 2018). However, sampling from wildlife populations in their natural environments unravels variation in ecologically relevant contexts (Amato, 2013). Controlled laboratory experiments, such as ours, can deepen our understanding of direct relationships observed in natural environments. Our study serves as an important link between EDCs (e.g., xenoestrogens) and gut microbiota and exemplifies how future research might expand upon the influence of such changes on health of wildlife populations.

AUTHOR CONTRIBUTIONS

Kaitlyn M. Murphy, Daniel A. Warner, Mary T. Mendonça, John W. Finger, and Meghan D. Kelley contributed to the inception and development of the experiment. Kaitlyn M. Murphy collected and analysed all data as well as wrote the first draft of the manuscript. Madison M. Watkins aided in caring for the alligators and in reviewing the manuscript. John W. Finger and Meghan D. Kelley assisted with caring for and advising alligator housing, assisting in alligator transport from LA, and in reviewing drafts of the manuscript. Ruth M. Elsey supplied the alligators and assisted with drafting the manuscript. Daniel A. Warner and Mary T. Mendonça contributed greatly to writing the manuscript.

ACKNOWLEDGEMENTS

Special thanks to M. Liles for allowing us to use his laboratory to perform DNA extractions and to A. Howard and T. Glenn for performing all Illumina sequencing in the lab at UGA. Thanks to A. Hoffman, H. Hallowell, K. Higgins, J. Ha, J. Mauldin, T. Cook, O. Schweikart, and A. Fargevieille for their assistance on this project. Funding was provided by the IUCN-SSC Crocodile Specialist Group, the Auburn University Museum of Natural History, and the Auburn University Department of Biological Sciences.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under Accession Number PRJNA831851. All scripts and data can be accessed via the following GitHub account: https://github.com/kmm0155/Alligator-Gut-Microbiome-Murphyetal

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Murphy, K.M., Watkins, M.M., Finger, J.W. Jr., Kelley, M.D., Elsey, R.M., Warner, D.A. et al. (2022) Xenobiotic estradiol-17ß alters gut microbiota of hatchling American alligators (*Alligator mississippiensis*). *Environmental Microbiology*, 24(12), 6336–6347. Available from: https://doi.org/10.1111/1462-2920.16222