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Increasing Alligator Snapping Turtle Head-Starting Success through Housing Enrichment and Inoculation of Hatchlings with Digestive Microbiota

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INCREASING ALLIGATOR SNAPPING TURTLE HEAD-STARTING SUCCESS THROUGH HOUSING ENRICHMENT AND INOCULATION OF HATCHLINGS WITH DIGESTIVE MICROBIOTA

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Master of Science, Biology

By

Kristen Erin Sardina

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ABSTRACT

Due to historic overharvest and habitat degradation, the alligator snapping turtle (*Macrochelys temminckii*) has experienced population declines throughout its range. Tishomingo National Fish Hatchery in southern Oklahoma began a captive head-start program for this species in 2000 and has since released over 1,400 turtles in the region. However, there has been a recurring trend of turtles growing faster after release than while in captivity. My research sought to investigate this pattern by determining: 1) the influence of housing enrichment and housing density on juvenile growth rates and stress in indoor enclosures, and 2) the effects of supplementing hatchlings with adult feces to enhance the gut microbiome. I found that the presence of floating mats in indoor tanks improved growth rates compared to other structural components. Group density did not affect average growth rates, but animals that were housed communally exhibited more variable growth than individuals housed without conspecifics present. When exposed to feces of adult conspecifics, hatchlings assimilated fiber components of their omnivorous diet more efficiently than those exposed only to deionized water or creek water. Based upon these results, flotant structures and exposure to microbes found in the feces of adult *M. temminckii* are recommended for future head-start efforts.

KEYWORDS: *Macrochelys temminckii*, head-starting, captive husbandry, microbiome, growth

This abstract is approved as to form and content

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Day B. Ligon, PhD
Chairperson, Advisory Committee
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OVERVIEW

Turtles tend to be long-lived, slow-growing, and have delayed sexual maturity, and these life-history traits pose unique challenges for mitigating population declines. Young turtles often experience high mortality rates during the first years of life; therefore, population bottlenecks — especially those impacting reproductive-age adults — frequently result in detrimental delays of recovery (Congdon et al. 1993). Head-start programs attempt to alleviate this problem by breeding and raising animals in captivity to a size that presumably increases their likelihood of survival upon release into the wild (Heppell et al. 1996).

The alligator snapping turtle (*Macrochelys temminckii*) is currently listed as 'Vulnerable' by the IUCN Red List (1996), chiefly due to historical overharvest, impoundments, and habitat alteration. Tishomingo National Fish Hatchery in southern Oklahoma implemented a head-start program for alligator snapping turtles in 2000. Despite evidence of the program's general success, sub-optimal growth rates of *M. temminckii* in captivity compared to those after release (Moore et al. 2013; Anthony 2015) begs the question of what the turtles encounter in the wild that is unavailable to them in captivity.

In Chapter 1, I examine the effect of species-specific probiotic supplementation on growth rates, digestive efficiency, and microbial community composition in *M. temminckii* hatchlings. The experiment described in this chapter tracks the intake, output, and assimilation of an omnivorous diet. Fecal samples collected at the beginning and end of the experiment were analyzed for their microbial community composition using 16S

rRNA gene sequencing. These differences in communities were used to make inferences about the impacts of endogenous communities on hatchling growth and digestion.

Chapter 2 examines the influence of housing structural enrichment and housing density on growth rates and stress hormone levels in juvenile *M. temminckii* at Tishomingo National Fish Hatchery. The housing enrichment experiment in this chapter tests the application of four different structural arrangements in indoor turtle tanks. Structures were constructed to mimick habitats *M. temminckii* has been reported to associate with in the wild. The housing density experiment involved housing turtles low-, medium-, and high-density conditions. In both experiments, growth rates and corticosterone concentrations were measured at the beginning and end of the experiments' duration to test the effects of different treatments.

THE ROLE OF GUT MICROBIAL SYMBIONTS IN ALLIGATOR SNAPPING TURTLE HATCHLING GROWTH AND DIGESTIVE EFFICIENCY

Abstract

The ability for vertebrates to exploit plant matter for energy largely relies on complex associations with fermentative microbes. Coprophagy as a behavioral means of microbial acquisition and maintenance has been observed most frequently in mammals; however, there is growing evidence that herbivorous reptiles associate with kin or conspecifics for brief periods in their lives to obtain species-specific microbes that are functionally tailored to their diet. Despite specializations for carnivory that include a sharp, recurved beak and a unique lingual lure, the Alligator Snapping Turtle (*Macrochelys temminckii*) is a dietary generalist that frequently consumes aquatic vegetation and detritus. Based on their dietary habits, I hypothesized that providing hatchlings with adult feces upon hatching would improve digestive efficiency and growth. As predicted, assimilation efficiency of neutral detergent fiber was significantly greater in turtles supplemented with feces than those exposed to either creek water or deionized water, but there was no difference among groups in assimilation of acid detergent fiber or crude protein. Growth rates were equal among treatments, although the short duration of the experiment may have been insufficient for differences in size to emerge. The microbial community structure of *M. temminckii* hatchlings differed between the start and end of the 42-day experiment. Inoculation material had no effect on the turtles' core microbiomes at the end of the experiment; however, hatchlings provided with adult conspecific feces had higher abundances of fermentative bacterial groups.

Introduction

Fermentative microsymbionts in the vertebrate gut are often credited for allowing the evolution of obligative and facultative herbivory in vertebrates (Ley et al., 2008; Stevens and Hume, 1998). In fact, most multicellular organisms are genetically incapable of producing cellulase enzymes to hydrolyze cellulose in plant cell walls; therefore, the synthesis of endogenous cellulase by microbes is necessary for efficient digestion and nutrient extraction. Exclusively in the presence of gut microbial communities, a host's digestive process may include the fermentation of cellulose and lignin into digestible nutrients used for energy in the form of fatty acids, conversion of nitrogenous compounds into ammonia and microbial proteins, production of B vitamins, and competitive interactions between commensal and pathogenic bacteria (Stevens and Hume, 1998; Pough et al., 2013; Hanning and Diaz-Sanchez, 2015). Despite their general importance to digestive processes, gastrointestinal microbiomes can vary markedly in species richness and abundance, and community patterns often correlate with diet (Wu et al., 2011), digestive system morphology (Stevens and Hume, 1998), the external environment (Bright and Bulgheresi, 2010), and phylogenetic history (Ley et al., 2008) of a host.

The ubiquity of the symbiotic relationship between a host and its gut microbiome becomes disjointed when broad patterns in gut microbiomes align with the digestive strategies of vertebrate hosts, which vary with diet, anatomy, physiology, and energetic demands. For example, foregut-fermenting vertebrates are characterized by a fermentation chamber that is proximal to the small intestine in an enlarged forestomach. This facilitates the digestion of microbial mass for additional protein, in which amino

acids are subsequently absorbed in the small intestine. In contrast, hindgut-fermenting vertebrates delay microbial fermentation and absorption of synthesized nutrients until fiber passes through a single-chambered stomach and reaches the enlarged large intestine and cecum; this strategy results in undigested microbes that are excreted in the feces. Hindgut fermentation is employed in most herbivorous species of reptiles, mammals, and birds (Stevens and Hume, 1998; Mackie, 1999). Delayed fermentation and consistent excretion of gut microbes in feces (Ley et al., 2008), along with the rapid pace at which food is digested (Fletcher et al., 2010) results in reduced energetic gain of hindgut fermenters by up to half in comparison to ruminants (Walter, 2011).

Research regarding microbe acquisition by a host and the impacts of the complex symbiotic relationships between a multicellular organism and its gut microbiome is in its infancy (Kostic et al., 2013). The most frequent means of acquiring and maintaining gut microbial communities is proposed to occur through environmental sources, collectively termed 'horizontal transmission' (Lombardo, 2008; Sanders et al., 2014; Yuan et al., 2015). Horizontal transmission may be achieved via regurgitation of food items from parents to offspring (van Dongen et al., 2013), grooming of kin (Ley et al., 2008), fluid exchange during copulation (White et al., 2011), acquisition from the nest environment (Benskin et al., 2015; Kohl, 2012), and coprophagy (Funkhouser and Bordenstein, 2013). Coprophagy is a behavior in which organisms obtain endogenous microbes by intentionally consuming feces from itself, kin, conspecifics, or heterospecifics. This behavior may serve as a method to re-digest contents for further nutrient extraction, consume excreted microbes for protein, (Demment and Soest, 1985), re-establish impaired microbiomes (Zimmerman and Tracy, 1988), or allow for microbial

colonization in neonates (Troyer, 1984). Ingestion of soil and the use of coprophagy to acquire microbes from kin has been described in *Iguana iguana* neonates in the wild; this study also reported that inhibiting these behaviors resulted in diminished growth rates (Troyer, 1984).

To date, over 90% of microbial symbiont research has focused on mammalian hosts, which comprise only 8% of vertebrate species (Colston and Jackson, 2016). While most mammalian taxa exhibit parental care and sociality to some degree, this life-history strategy is less common among reptiles. Fewer interactions with kin and conspecifics would presumably reduce the time span and/or opportunities for reptile neonates to establish microbiomes via coprophagy from these interactions in the wild. Aquatic turtles are particularly difficult to study, as they could indirectly and inadvertently acquire water-borne feces from conspecifics. The influence of microbial communities on digestion in freshwater turtles have been described in *Pseudemys nelsoni*, *Trachemys scripta*, *Emys orbicularis*, *Trionyx sinensis*, *Sternotherus odoratus*, and *Dermatemys mawii*, representing four of the nine extant turtle families (Bjorndal and Bolten, 1990; Bouchard and Bjorndal, 2005; Zhang et al., 2014; Nowakiewicz et al., 2015; Rawski et al., 2016; Rangel-Mendoza et al., 2018).

The alligator snapping turtle (*Macrochelys temminckii*) is omnivorous and a dietary generalist in the family Chelydridae. This species possesses a unique lingual lure to attract aquatic animal prey, and also consumes mammals, birds, and other turtles; however, plants and plant parts, including acorns, wood, and plant tubers consistently comprise a large proportion of gut contents (Sloan et al., 1996; Elsey, 2006; East and Ligon, 2013). The roles of coprophagy and microbial fermentation are currently

unexplored in this species. Fast growth is exceedingly important in alligator snapping turtles due to high predation rates of juveniles in the wild prior to exceeding predator gape limitations (Dreslik et al., 2017), size-dependent sexual maturity, and the positive relationship between body size and reproductive output in females (Tucker and Sloan, 1997; Thompson, 2013).

This study sought to determine whether exposure of *M. temminckii* hatchlings to adult feces influenced endogenous microbial community composition, digestive efficiency, and growth. I hypothesized that captive alligator snapping turtle hatchlings that are exposed to microbes shed by adult conspecifics would have faster growth rates, improved digestive efficiencies, and more diverse gut microbial communities than turtles only exposed to creek water or deionized water.

Methods

My experiment took place from 23 September – 4 November 2016 at Missouri State University. I acquired hatchling Alligator Snapping Turtles from a captive head-start program at Tishomingo National Fish Hatchery in southern Oklahoma. Hatchlings were acquired from three clutches and all hatched between 21 – 23 August 2016. Prior to transport to the university on 2 September 2016, the hatchlings and their eggs remained in environmental chambers at 28° C in the plastic boxes in which they hatched. Boxes contained damp vermiculite (1:1 water:vermiculite, by mass) and dividers between each turtle to limit physical contact with clutch-mates and maintain identity with its egg and clutch. Upon arrival at to the lab, I gently cleaned each hatchling to remove any vermiculite or egg debris with a soft-bristled brush. I then allowed the turtles to dry before marking them with numbered/color-coded tags adhered to the carapace (Queen

Marking Kits; The Bee Works, Oro-Medonte, Ontario, Canada). I housed each turtle singly in 1.9-L clear polycarbonate containers (CamSquare 2SFSCW; Cambro Manufacturing, Huntington Beach, CA, USA) filled with 400 mL of deionized water; this volume allowed turtles to surface easily to breathe (Appendix A). I distributed containers between two environmental chambers maintained at 28°C with a 12-hour light cycle (Appendix B). Upon placement into their assigned containers, I allowed the hatchlings to acclimate for 24 days before feeding was initiated. This time was necessary for turtles to complete assimilation of residual yolk prior to their first feeding. During this period, each turtles' first post-hatching fecal pellet was collected and stored in a 0.5-mL polypropylene micro centrifuge tube, and then frozen at -20 °C. To eliminate potentially confounding effects of microclimate variation, I randomly repositioned hatchlings within and between the two environmental chambers weekly.

Each container housing a hatchling was labeled to identify the turtle it contained and the group to which it was assigned. I supplemented each hatchlings' food weekly throughout the study with 1-mL of material corresponding with the control or treatment group to which they were assigned. Hatchlings were assigned to one of three groups: the control group consisted of hatchlings that were provided food inoculated only with deionized water, and thus were not exposed to any sources of microbes to which the experimental groups were not equally exposed. The first treatment group was provided with food inoculated with creek water, while the second treatment group was administered food inoculated with feces obtained from adult conspecifics. The creek water was collected from Pennington Creek, located in southern Oklahoma. It is spring-fed and free of resident Alligator Snapping Turtles. I harvested feces from captive—but

formerly wild-caught—adult turtles that are maintained in outdoor ponds at Tishomingo National Fish Hatchery. These turtles serve as brood-stock for a head-start program and were confiscated from an illicit turtle farm in 2006. Therefore, the duration of time they were kept in captivity was unknown.

After acclimation of the hatchlings was completed, I recorded morphometrics weekly for each hatchling, including mass (± 0.01 g), straight midline carapace length (± 0.1 mm), carapace width (± 0.1 mm), straight plastron length (± 0.1 mm), and shell height (± 0.1 mm). I also began feeding a pre-measured mass of omnivorous reptile pellets (Mazuri Herbivorous Reptile LS Diet, PMI Nutrition International LLC, Richmond, IN, USA) at 0900 h each day. Food quantities were increased uniformly for all turtles as the experiment progressed and turtles grew, ensuring that small quantities of orts still occurred without compromising water quality. At 1200 hours, I removed orts from each container and noted whether it appeared that turtles had consumed any food. To minimize degradation of fecal samples as well as the mixing of food and waste, I always collected feces prior to feeding, after collecting orts, or at any other point that fecal waste was observed. Feces and orts collected between each hatchlings' first and final fecal excretions were stored separately but cumulatively for each hatchling in 50-mL centrifuge tubes in a -20 °C freezer. I rinsed and replaced water in housing containers every other day, or as-needed based on cleanliness. To prevent unintentional bacterial accumulation, I scrubbed housing containers weekly with 0.5% chlorhexidine gluconate solution. As a control to monitor changes in food mass, I conducted feeding, cleaning, and orts collection procedures in three additional containers that did not house hatchling turtles. At the conclusion of the experiment, I gave hatchlings several days to excrete all

waste and stored the final fecal sample in a 1.5-mL microcentrifuge tube. These, and each turtles' initial fecal samples were used to measure differences in microbiomes among treatments. Prior approval for this project was obtained from the Missouri State University Institutional Animal Care and Use Committee (Approved: 9/2/16; IACUC ID 17-004.0).

Digestive Processing Methods. All samples were sent to Colorado State University for analysis. There, proportions of acid detergent fiber (ADF), neutral detergent fiber (NDF), crude protein (CP) and nitrogen (N) were quantified for food, orts, and feces samples (measured in 0.1-g aliquants). NDF represents all cell wall components of plants (cellulose, hemicellulose, lignin and cutin), and ADF represents the lignocellulose and cutin components that characterize woody plant tissues. Samples were oven-drying 60 °C and dry weights were recorded. NDF and ADF were determined using the Ankom^{200} Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY) and the associated manual's guidelines (ANKOM Technology, 2017a,b). The AOAC (2006) Official Method 992.15 (TruSpec CN Carbon/Nitrogen Determination Instruction Manual, December 2004, Leco Corp., St. Joseph, MI) described in Desimone et al. (2013) was used to obtain proportions of CP. Sample aliquants were weighed in aluminum combustion tins. A standard reference of ethylenediaminetetraacetic acid (EDTA—9.75% nitrogen) was used to establish calibration and blanks after every 25 samples run. After samples were optimized, crude protein of each sample was calculated by multiplying total nitrogen content by 6.25.

Digestive Assimilation Calculations. Digestive assimilations were calculated for each hatchling using the following equation (Bjorndal, 1987; Durstche, 2004):

$$\frac{\text{Intake of } X - \text{Output of fecal } X}{\text{Intake of } X} \times 100$$

where intake and output are measured in grams, and X was any diet component in a given sample (e.g., NDF). To calculate the total intake by each turtle, I subtracted the total dry mass of orts from total dry mass of food administered to each turtle. Intake and output values of each nutrient were substituted into the above equation.

Statistical Analysis: Growth and Digestive Assimilation. Preceding comparison tests across groups, I checked distributions of initial morphometrics, growth rates, and nutrient amounts and assimilations for normality using Shapiro-Wilk tests; I then used Kruskal-Wallis tests when data failed the assumption of normality. Before the experiment was initiated, I determined that the variance of turtle morphometrics was the same across treatments using a one-way analysis of variance (ANOVA). To account for variation in initial hatchling body size, I calculated size-corrected growth rates in $mg \cdot g^{-1} \cdot day^{-1}$. I then compared experiment growth rates across treatments using a Kruskal-Wallis test, and compared digestive assimilations using either ANOVAs or non-parametric Kruskal-Wallis tests.

Microbial Community Structure. A fecal sample was taken from each individual at the onset of the study and at the end of the 42-day experiment. The fecal microbiota of Alligator Snapping Turtle hatchlings in each of the three nutritional groups were examined by high throughput next-generation Illumina sequencing. Initially, these data were used to compare microbial population differences between nutritional groups. Total DNA was extracted using a QiaAMP DNA Stool mini kit (Qiagen, Germantown, Maryland). The V4 variable region were amplified using the 515F/806R primers with a barcode addition to the forward primer using the HotStarTaq Plus Master Mix Kit

(Qiagen, Germantown, Maryland) under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step at 72 °C for 5 minutes was performed. Samples were pooled in equal proportions based on their molecular weight and DNA concentrations and were purified using Ampure XP beads. Then the pooled and purified PCR product was used to prepare an Illumina DNA library and sequenced at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using the Illumina MiSeq platform following the manufacturer's guidelines. The derived Q25 sequence data was processed using Qualitative Insights Into Microbial Ecology (QIIME) 1.9. In brief, sequences were joined, depleted of barcodes, and sequences <150bp or with ambiguous base calls removed. Sequences were denoised, OTUs generated, and chimeras removed. Operational taxonomic units (OTUs) were taxonomically classified using BLASTn against a curated database derived from Greengenes v13.5 reference database (DeSantis et al. 2006) using a >97% identity cutoff to classify at the species level (Ritari et al., 2015). These data were then used to estimate alpha and beta diversity, compositional differences between nutritional groups, and temporal differences within nutritional groups.

Normalized QIIME OTU data were used for Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to examine differences in metabolic function and chemical pathways of microbial communities across treatment groups (Langille et al., 2013). KEGG Orthology (KOs) were used to predict metabolic and functional capabilities of the identified microbes.

Statistical Analysis: Microbiome Structure. The 16S rRNA gene sequences

were processed using QIIME v1.9.1+dfsg pipeline (Caporaso et al., 2010) and Bio-Linux 1.8 (Field et al., 2006) to determine the diversity and composition of that fecal-associated microbiota using default parameters. Differences in microbial community composition across treatments (beta diversity) were assessed using phylogenetic metrics (weighted and unweighted UniFrac distances) (Lozupone and Knight, 2005; Lozupone et al., 2011). Ordination plots were visualized using EMPeror (Vázquez-Baeza et al., 2013).

Significant differences in taxonomic richness were assessed across treatments using the nonparametric Kruskal-Wallis test and between treatments using a t-test. Significant differences in the relative abundances of individual bacterial taxa across treatments or factor levels were determined using ANOVA and the false discovery rate (FDR) correction. T-tests were used when comparing the relative abundances of individual taxa between two treatments.

Results

Growth. I had initially randomly assigned 60 Alligator Snapping Turtle hatchlings from three clutches to my three treatments; however, 26 of those animals were excluded from analyses due their latency to begin feeding, presumably a result of prolonged assimilation of residual yolk. In addition, one hatchling died during the acclimation period for my experiment. This hatchling retained an unusually domed carapace until its death; this condition is common among hatchling Alligator Snapping Turtles as they unfold from their eggs; however, failure to attain a typical morphology suggests that this hatchling may have been congenitally deformed.

Morphometrics of hatchlings at the beginning of the experiment were equal across treatments. Mass averaged 18.38 ± 0.14 g ($F_{2,30}$ = 0.24, P = 0.787), straight mid-line carapace length (SCL) averaged 38.80 ± 0.17 mm (H = 1.51, P = 0.470), and plastron length (PL) averaged 30.41 ± 0.12 mm ($F_{2,29}$ = 0.143, P = 0.867). The average 42-day growth rate of hatchlings was 3.17 ± 0.27 $mg \cdot g^{-1} \cdot day^{-1}$ (range = 1.90–6.34 $mg \cdot g^{-1} \cdot day^{-1}$) (Table 1) and did not differ significantly among treatments (H = 0.960, P = 0.619); however, while not statistically significant, the group inoculated with feces maintained the highest weekly average mass and straight midline carapace length throughout the experiment (Figure 1).

Digestive Efficiency. Due to some hatchlings' latency to feed, 26 turtles were removed from digestive assimilation analyses (Table 1). The dry mass of food distributed to turtles across treatments was the same over the duration of the study ($F_{2,30}$ = 1.690, P = 0.202), as was the total dry matter intake ($F_{2,30}$ = 0.0538, P = 0.948) (Table 1). I adjusted the values for the mass of turtle food distributed, intake, and nutritional composition, to adjust for soluble components and moisture that were lost from food during the process of soaking and oven-drying. The assimilation of total dry matter by Alligator Snapping Turtle hatchlings in my experiment was affected by treatment group ($F_{2,30}$ = 9.636, P < 0.001). Turtles supplemented with adult feces had greater assimilation efficiencies of dry matter than those given deionized water (P < 0.001) and those given creek water (P = 0.031). Due to the fact that small quantities of feces were recovered from some hatchlings, in some cases only NDF and ADF could be analyzed. Therefore, crude protein analyses were conducted on a subset of samples: deionized water (n = 9), creek water (n = 9) and adult feces (n = 4). Assimilation efficiency of crude protein did not

differ among treatment groups ($F_{2,19} = 0.117$, $P = 0.890$; Figure 2A). Assimilation efficiencies of lignocellulose (ADF) were also influenced by the source of microbiota provided to turtles ($F_{2,30} = 5.689$, $P = 0.008$; Figure 2B). Turtles exposed to adult feces displayed improved ADF assimilation compared to turtles exposed to DI water ($P = 0.006$). I did not observe these differences between turtles given feces versus those given creek water ($P > 0.05$), or between the creek water group and the deionized water group ($P > 0.05$). The ability for hatchlings to assimilate cell wall contents (NDF) was also enhanced in turtles that were inoculated with adult feces ($H = 6.158$, $P = 0.046$; Figure 2C); however, non-parametric pairwise comparisons failed to identify treatment differences (all $P > 0.05$).

Endogenous Microbial Communities. After trimming barcodes and primers and removing chimeras, I generated a total of 4,900,245 sequence reads that represented 27,698 OTUs. These OTUs were concentrated in 9.7% of the 70 samples used for analyses. The dominant phyla across the three treatment groups consisted of mainly Proteobacteria, Bacteroidetes, and Firmicutes (Figure 3). The alpha diversites of treatments and controls in my experiment differed across groups (Table 2) and controls (Figure 4). Hatchlings supplemented with adult feces contained greater average microbial taxonomic richness than those supplemented with creek water ($t = -4.93$; $P = 0.014$) or deionized water ($t = -7.34$; $P = 0.014$); however, differences were not detected between turtles provided with creek water and deionized water ($t = 2.51$; $P = 0.34$) (Figure 4). The combined initial samples of turtles had significantly lower average taxonomic richness than those who received creek water ($t = 3.47$; $P = 0.042$) or adult feces ($t = -8.31$; $P = 0.014$) in my 42-day trial, whereas I did not observe this pattern in comparison to turtles

that only received deionized water ($t = 0.69$; $P = 1.0$). Interestingly, the creek water used for inoculation had the greatest average taxonomic diversity overall, and the turtles given feces possessed a greater richness than was distinguished in the feces control sample (Figure 4).

The core microbiomes of hatchlings at the beginning and at the end of the 42-day trial clustered distinctly from each other in principle coordinate analysis (PCoA) plots, based on weighted and unweighted UniFrac distances (Figure 5). Additionally, the turtles that were exposed to adult feces distally clustered within the treatment cluster. Hatchlings within this treatment group had the highest abundance of phylum Firmicutes (22.2%) compared to the turtles early on in the experiment (9.7%), those supplemented with creek (15.0%) or deionized water (18.3%), the adult feces control (5.7%), and the creek water control (0.5%). Especially prominent in the Firmicutes phylum was the appearance of family Ruminococcaceae, well known for its ability for its fibrolytic activity (Flint et al., 2012), that initially made up 1.9% of the OTUs. After 42 days, abundances rose in all exposure groups, but was found at the highest levels in turtles exposed to adult conspecific feces (6.9% of the OTUs). In comparison, Ruminococcaceae was found in creek water-exposed turtles at 2.5% and DI exposed turtles at 3.0%. Ruminococcacea was almost absent from creek water (0.1%); however, adult feces contained 1.5% abundance.

Discussion

Growth rates of Alligator Snapping Turtles in the digestive efficiency experiment were not significantly influenced by treatment groups; however, there was a non-

significant trend for turtles provided with adult feces to rapidly achieve and then consistently maintain the greatest average mass and carapace length. Troyer (1984) reported that hatchling *Iguana iguana* given adult feces only exhibited significant increases in growth after spending several weeks consuming soil in the nest, followed by the consumption of conspecific feces at 3–5 weeks of age. Iguanas that were not given conspecific feces during this time span grew more slowly, as did those given feces prior to three weeks of age. This suggests that if *M. temminckii* hatchlings are performing coprophagy in the wild as a means of microbial establishment, it could be a time-sensitive event (e.g. time after yolk assimilation), one that is effectively triggered by some environmental cue, or a succession of microbes. Alligator Snapping Turtle hatchlings will remain in the nest chamber for as long as three weeks (Holcomb and Carr, 2011), creating ample opportunity for similar means of generalized microbial acquisition.

Coprophagy has been inferred to serve as an important path for microbial acquisition and maintenance of diverse and physiologically functional microbial communities in numerous vertebrate taxa. In my study, Alligator Snapping Turtle hatchlings provided with adult conspecific feces shortly after hatching showed improved digestive assimilations of combined lignocellulose (ADF) compared to those given creek water or deionized water. This trend was also evident for digestion of the the digestion of total cell wall components (NDF) by hatchlings given feces, but pairwise testing failed to indicate significance, possibly due to uneven and low sample sizes that decreased statistical power. The significant increases of lignocellulose breakdown is especially notable due to its high indigestibility relative to all other cell wall components. As lignocellulose content increases in a diet item, the digestibility of that item decreases, and

requires the service of specialized fermentative microsymbionts (Zimmerman and Tracy, 1988). Amorocho and Reina (2008) reported this to be true in *Chelonia mydas agassizii* that subsisted on diets of leaves, fish, and fruits. Dry matter assimilation of turtles in my experiment were intermediate to those in a digestive experiment containing two groups of *Pseudemys nelsoni* hatchlings characterized by diets of shrimp or duckweed (Bouchard, 2004); both groups were inoculated with adult conspecific feces at the start of the experiment. Adult *Trachemys scripta scripta* fed an omnivorous diet of *Tenebrio* larvae and duckweed had lower assimilations of dry matter than *M. temminckii* hatchlings by >10%, which may be attributed to the lower digestibility of whole duckweed leaves or larger bite size of adult *T. scripta scripta* (Bjorndal, 1991; Bjorndal and Bolten, 1992). Nonsignificant trends for greater NDF and ADF assimilation in my experiment were also shown in hatchlings inoculated with creek water compared to those given deionized water. This is presumably due to the advantage of having exposure to broad, environmentally-available microbes (Troyer, 1984; Ley et al. 2008); however, the acquisition of species-specific, or specialized fermentative microbes has been described to be more impactful for growth, digestion, and microbial community development in reptiles (Troyer, 1982). Additionally, crude protein was assimilated equally by hatchlings in all three groups at rates that were consistently over 90%.

Core phyla of final fecal samples were very similar to other studies of herbivorous Testudines (Gaillard, 2014; Zhang et al., 2014; Yuan et al., 2015; Abdelrhman et al., 2016; Price, 2016). This may be due, in part, to the similar physiological constraints that turtles and tortoises place on the environment of their microsymbionts, including gut morphology, location of the fermentation chamber, diet, and the fluctuating conditions

associated with the ectothermic condition (e.g. body temperature, metabolic rate, fasting, dehydration) (Gaillard, 2014; Hanning and Diaz-Sanchez, 2015; Colston and Jackson, 2016). Hatchlings supplemented with only deionized water also presented shifts in their microbial community composition. This could be attributed to the consumption or absorption of microbes in vermiculite or eggshell fragments through the mouth or residual yolk after hatchling, along with other sources of potential unintended cross-contamination. Another method of microsymbiont colonization may be obtained through vertical transmission. In amniotes, this method of transmission would consist of the transmission of indigenous bacteria from the female to her eggs during oviposition (Benevides de Morais et al., 2010).

The most dominant phylum represented across my experimental groups was Proteobacteria. Proteobacteria are recognized as a core contributor to most vertebrate microbiomes; however, the relative abundance of this group is disproportionately high for the *M. temminckii* hatchlings in my study compared to other reptiles, excluding sea turtles that are exposed to high abundances in saltwater (Keenan and Elsey, 2015; Price 2016). The unusual dominance of Proteobacteria in my study may be a result of immature gut microbiomes (Hanning and Diaz-Sanchez, 2015). Proteobacteria are efficient colonizers for the fasting or sterile gut, but are poor competitors; therefore, they are often replaced by more diet-specialized groups of anaerobic bacteria over time (Costello et al., 2010; Hanning and Diaz-Sanchez, 2015). This is supported by findings of Troyer (1984), in which iguanas consumed soil in the nest during the first several weeks of life, suggesting preparation of the gut for supplementation of fermentative microbes via coprophagy shortly after (Troyer, 1984). Alternatively, elevated abundance of Proteobacteria may

have been attributed to ambient environmental exposure, particularly by creek water, in which case this phylum may have been chiefly transient across hatchlings in my study. This is supported by the greater relative abundances observed in the adult feces control and creek control samples, as well as the lower abundances in hatchlings only inoculated with deionized water.

The next most abundant phylum was Bacteroidetes, which is also increasingly common in the gastrointestinal tract of carnivorous and herbivorous humans, vertebrates animals, and reptiles specifically (Ley et al., 2008; Costello et al., 2010; Colston and Jackson, 2016). This implies that this group plays an important role in digestion (Yuan et al. 2015). Microbes in this phylum are known to be metabolically broad; however, the majority are reported to be anaerobic and sacchrolytic, and play an integral role in early degradation of cellulose and hemicellulose (Colston and Jackson, 2016).

M. temminckii inoculated with adult feces contained the greatest taxonomic diversity across tratments and the adult feces control sample. The feces-inoculated hatchlings also had the largest abundances of Firmicutes, which are abundant among all mammals and predominately function as metabolizers of polysaccharides (Hanning and Diaz-Sanchez, 2015). Turtles in the feces group contained the the highest abundances of the class Clostridia at the end of the experiment, which frequently metabolize carbohydrates (Colston, 2017). More specifically, they contained more than double the relative abundance of the family Rumminococcaceae compared to turtles that did not receive feces. This family is only currently found as a resident in the gut of herbivorous vertebrates and has fibrolytic capacity to create short-chain fatty acids for the host (Campos et al. 2018). Over the span of my 42-day experiment, shifts in microbial

community composition were distinguishable across all groups. This is potentially due to the stability of the adult feces compared to the developing hachling where the introduction of adult feces causes dramatic microbial changes as the resident gut microbiome matures (Yuan et al. 2015). This dramatic increase in diversity may help enrich fiber degraders like Ruminococcaceae, leading to better digestive efficiency of fiber.

Despite the recent explosion of technological capabilities and research to describe vertebrate gut microbiomes, the elusive lifestyle of most wild reptiles leaves room for speculation about how hosts acquire species-specific microbes. Inoculating Alligator Snapping Turtle hatchlings with adult conspecific feces improved digestion of cell wall components, and also showed trends for increased assimilation of lignocellulose (ADF) and growth. Supplementing hatchling food with feces also increased taxonomic richness, and resulted in higher abundances of microbial groups with sacchrolytic and fibrolytic functions. While behavioral observations of reptile neonates in the wild are rare, the ability to examine the influence of induced coprophagy may provide clues for how succession of microbiomes takes place. While there are several of these functional trends for greater fermentation capabilities in the feces-supplemented hatchlings, further divergence of this group may be time-dependent, warranting long-term monitoring of changes in growth, digestive efficiency, and microbial community assemblages.

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Table 1. Average initial mass, growth rates, and digestive processing of hatchling Alligator Snapping Turtles provided with deionized water, creek water, or adult conspecific feces in their food. Nutrient values are on a dry-matter basis. Values are ± 1 standard error for turtles of each treatment.

	Treatment Groups		
	DI Water (n = 11)	Creek Water (n = 14)	Feces (n = 8)
Initial Mass (g)	18.87 ± 0.15	18.73 ± 0.28	18.96 ± 0.22
Growth Rate ($mg \cdot g^{-1} \cdot day^{-1}$*)*	3.17 ± 0.27	3.07 ± 0.33	3.47 ± 0.35
*Intake (g)**	2.05 ± 0.06	2.03 ± 0.07	2.06 ± 0.12
NDF	0.75 ± 0.04	0.70 ± 0.03	0.71 ± 0.05
ADF	0.50 ± 0.01	0.50 ± 0.07	0.51 ± 0.03
Crude Protein**	0.57 ± 0.02	0.53 ± 0.02	0.49 ± 0.03
Output (g)	0.58 ± 0.02	0.54 ± 0.02	0.50 ± 0.03
NDF	0.43 ± 0.01	0.41 ± 0.19	0.37 ± 0.03
ADF	0.50 ± 0.01	0.50 ± 0.02	0.51 ± 0.03
Crude Protein**	0.06 ± 0.002	0.05 ± 0.002	0.05 ± 0.003
Assimilation (%)			
Dry Matter	71.85 ± 0.51	73.33 ± 0.65	75.71 ± 0.53
NDF	42.71 ± 1.83	41.97 ± 1.23	46.84 ± 1.05
ADF	39.31 ± 0.90	42.08 ± 1.21	45.84 ± 1.70
Crude Protein**	90.21 ± 0.35	90.00 ± 0.44	90.26 ± 0.16

*Neutral detergent fiber (NDF) represents the cell wall constituents of plants (cellulose, hemicellulose, lignin and cutin). Acid detergent fiber (ADF) represents the lignocellulose content.

**Crude protein analyses contained treatment sample sizes that differed from those listed in the headings: DI water (n = 9), creek water (n = 9), and feces (n = 4).

Table 2. Alpha diversity metrics for fecal microbial communities of hatchling Alligator Snapping Turtles exposed to deionized water, creek water, or adult conspecific feces in a 42-day digestive efficiency experiment. Initial fecal samples were collected at the start of the experiment. Values are ± 1 SD.

	Treatment Groups			
Indices	Initial	DI Water	Creek Water	Feces
Observed OTUs	1335 ± 276	1208 ± 160	1475 ± 238	2166 ± 205
Chao1	2581 ± 602	2719 ± 422	3244 ± 531	4500 ± 598
Faith's Phylogenetic Diversity	105 ± 17	109 ± 12	128 ± 19	169 ± 16

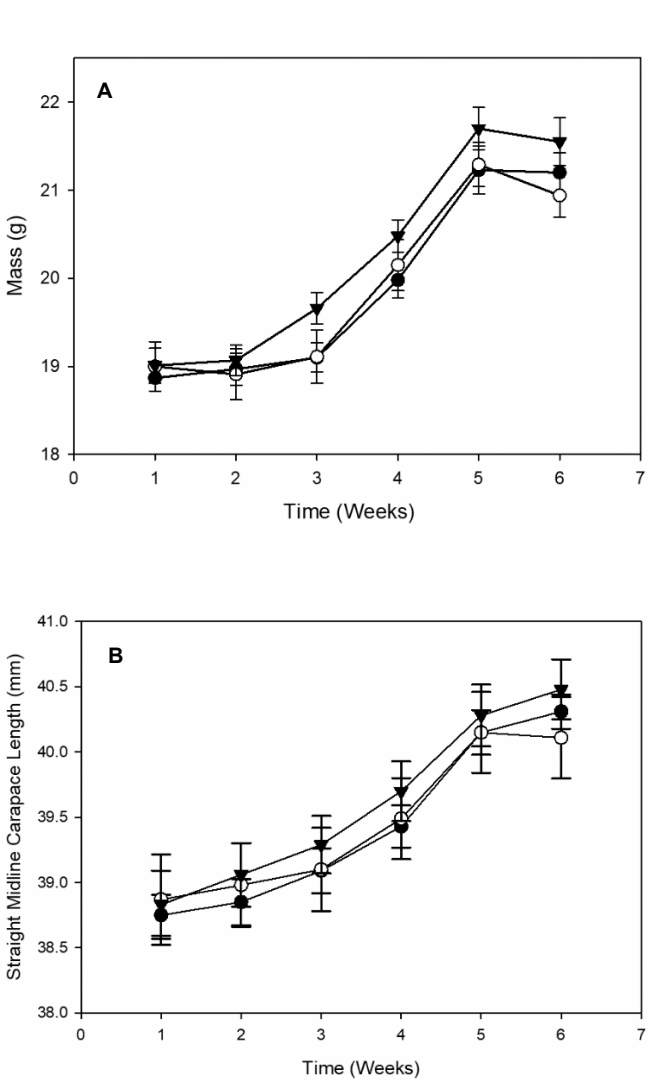

Figure 1. Average weekly A) mass and B) straight midline carapace length of Alligator Snapping Turtle hatchlings split into three groups characterized by the source of microbiota provided over a 6-week span. Closed circles = turtles inoculated with deionized water, open circles = turtles inoculated with creek water, and closed triangles = turtles inoculated with adult feces. Error bars are ± 1 SE.

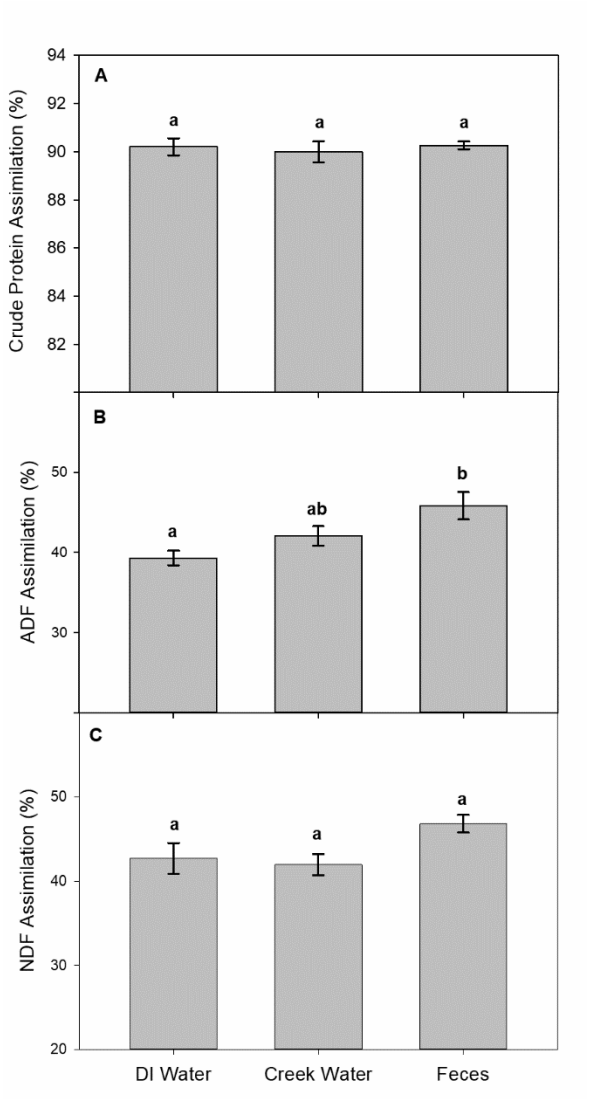

Figure 2. The assimilation efficiencies of A) crude protein, B) acid detergent fiber (ADF), and C) neutral detergent fiber (NDF) by *M. temminckii* hatchlings inoculated DI water, creek water, and adult *M. temminckii* feces. Lowercase letters illustrate the relationships among treatments for comparisons of the mean nutrient assimilation efficiencies. Error bars are ± 1 SE.

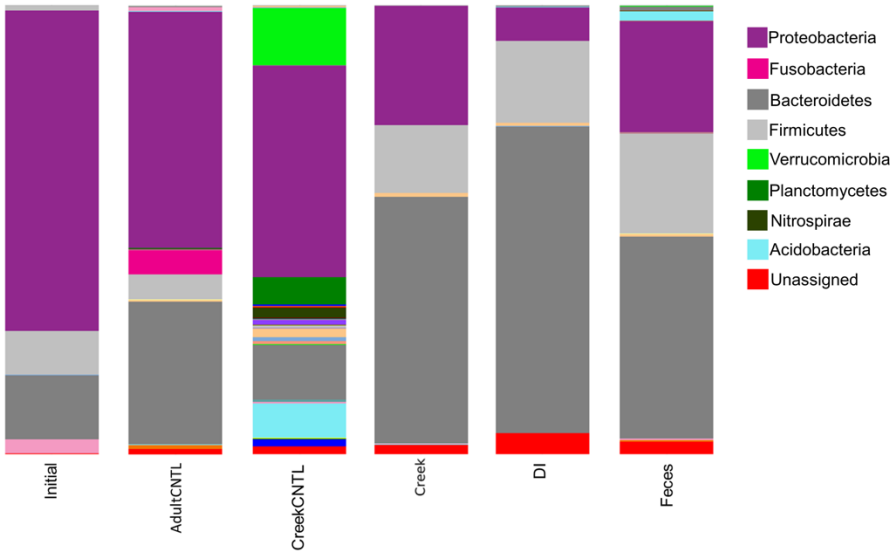

Figure 3. The relative abundance of core phyla in samples of adult feces, creek water, and hatchling feces at the start and end of a 42-day digestive efficiency experiment. Only major phyla are indicated for clarity.

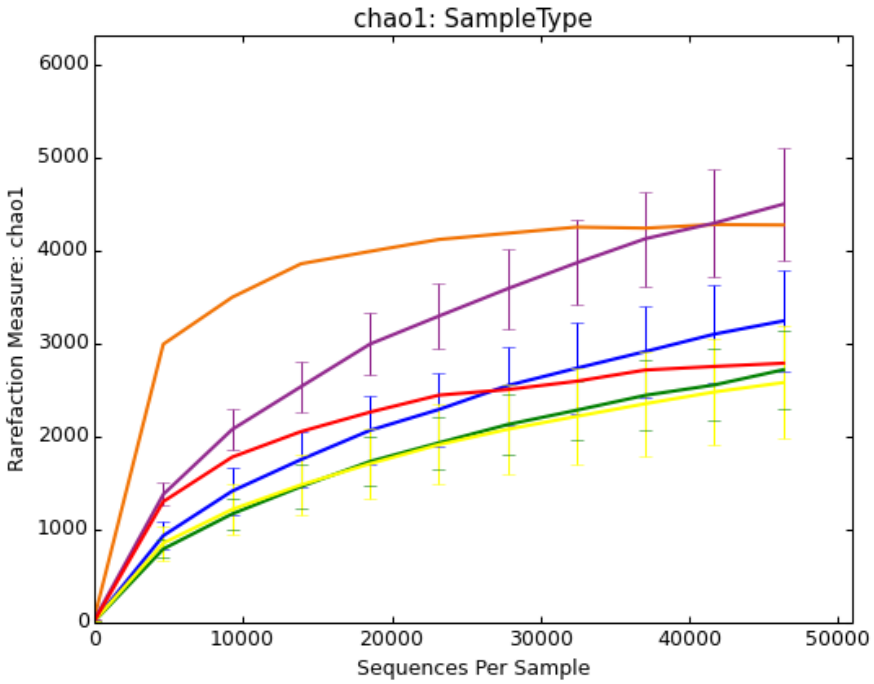

Figure 4. Rarefaction analysis of average operational taxonomic unit (OTU) richness (Chao 1) observed across gut microbiomes of *M. temminckii* hatchlings, as well as the creek water and adult conspecific feces of which they were inoculated. Error bars in control samples are not visible due to having proportionately fewer reads. Green = turtles inoculated with deionized water, yellow = combined turtles initial, red = adult conspecific feces control, blue = turtles inoculated with creek water, purple = turtles inoculated with adult feces, orange = creek water control.

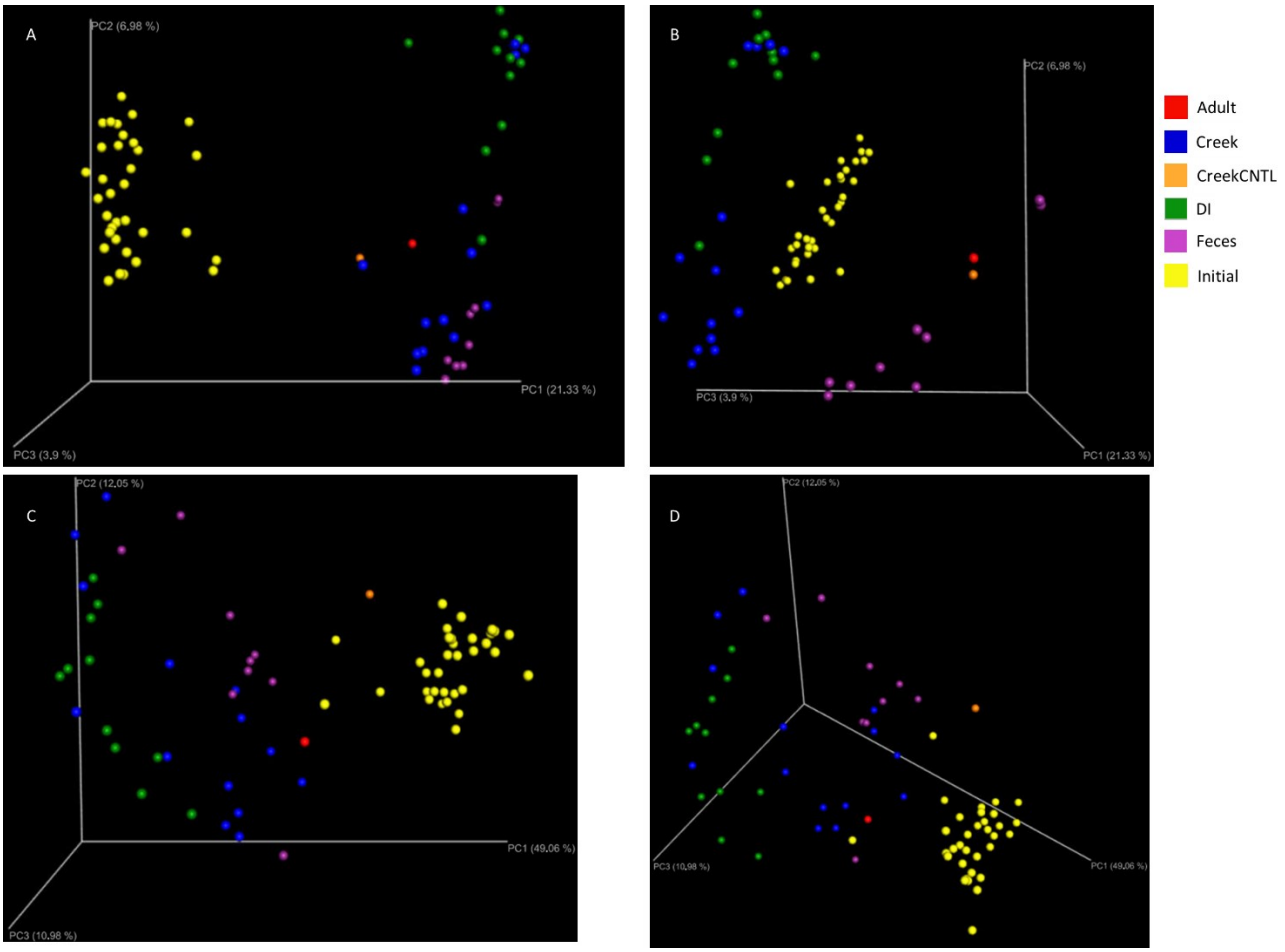

Figure 5. Principle coordinate analysis (PCoA) A-B) unweighted UniFrac distances and C-D) weighted UniFrac distances of representing the gut microbiomes of *M. temminckii* hatchlings in a digestive efficiency experiment, as well as the creek water and adult conspecific feces samples of which they were inoculated. Samples were rarefied to 46,329 reads. In all cases initial samples cluster as a distinct group. Within the treatment cluster, turtles that were exposed to adult feces clustered independently from the DI and creek water exposure (panels B and D).

EFFECTS OF INDOOR HOUSING CONDITIONS ON GROWTH RATE AND PLASMA CORTICOSTERONE LEVELS IN ALLIGATOR SNAPPING TURTLES

Abstract

The decline of alligator snapping turtle (*Macrochelys temminckii*) populations throughout its range has spurred efforts to propagate and head-start turtles in captivity, allowing them to reach a size less susceptible to mortality after release. Growth rates of captive juvenile *M. temminckii* at Tishomingo National Fish Hatchery have been generally lower than that after release; however, the underlying cause of retarded growth in captivity is unknown. Due to this species' cryptic nature and affinity for structure in the wild, I hypothesized that faster growth rates and reduced corticosterone levels would occur in enclosures with increased artificial refugia, as well as among turtles housed at lower densities. To test the effects of added structure, experimental treatments were designed to simulate observed habitat associations for *M. temminckii*, such as flotant vegetation, submerged structures, and overhead canopy cover. To determine the influence of housing density, treatments in a second experiment were manipulated based on conditions likely encountered in the wild, at the hatchery, and at densities that were considered comparatively crowded. Morphological measurements and blood samples were taken at the beginning and end of a 6-week experiment. Turtles maintained in enclosures with artificial flotant structures grew significantly faster than turtles maintained with other structural components. Stocking density had no significant effect on average growth over the duration of the study. Corticosterone levels were not

significantly different across treatments in either experiment. An important proximate result of my study will be improved head-starting practices for the species and, perhaps ultimately, will raise awareness for the implications of optimizing husbandry practices in conservation programs.

Introduction

While threats to reptilian taxa in freshwater ecosystems are reported to be disproportionately high, freshwater turtles are markedly at risk with 46–57% of species of conservation concern (Böhm 2013). Degradation of aquatic habitats occurs directly and indirectly from flood control structures, agricultural development, deforestation, draining of wetlands, mining, and proliferation of invasive species (Richter et al. 1997; Moll and Moll 2004). The need for captive wildlife propagation programs to augment or restore populations is rising. This has created an ongoing challenge for conservation biologists to better understand the ecology and life-history of declining species. Despite the recent expansion of knowledge for captive animal husbandry, reptiles remain relatively understudied, with much more research focused on mammals and anurans (Hayes et al. 1998; Bashaw et al. 2016). Historically, reptiles were often considered to be unresponsive to most environmental influence in captivity (Burghardt 2013), but few studies have conducted quantitative physiological assessments of housing conditions by measuring influences of the captive environment on growth, adrenal response, or reproductive success (Oonincx and Leeuwen 2017). However, a few studies have described broad effects of husbandry and captive environment on overall health of Chelonids. For example, Case et al. (2005) described broad effects of husbandry and captive

environment on overall health, finding that eastern box turtles (*Terrapene carolina carolina*) had lower heterophil-to-lymphocyte ratios when provided mulch substrate and hides, suggesting lower levels of stress. Additionally, Chen et al. (2007) found that captive soft-shelled turtles (*Pelodiscus sinensis*) exhibited lower growth rates when maintained at higher stocking densities.

The alligator snapping turtle (*Macrochelys temminckii*) is currently listed as 'Vulnerable' on the IUCN Red List (1996) due to habitat degradation as described above, along with historically unregulated harvest from the 1960s to the 1980s (Pritchard 1979a; Roman et al. 1999; Reed et al. 2002). Delayed sexual maturity and high early life-stage mortality rates have also exacerbated these bottlenecks, causing reverberating effects that have delayed resurgence and repopulation. These challenges are exacerbated by illegal poaching, despite legal protections throughout its range (U.S. Department of Justice 2017). Reintroduction of juveniles and translocation of adults from healthy populations are likely viable conservation measures; however, opportunities for translocation are rare because of the paucity of robust donor populations (Riedle et al. 2008; Moore et al. 2013). Captive propagation that includes growing hatchling *M. temminckii* to a larger size in captivity prior to reintroduction presumably gives these animals a greater likelihood of survival upon release (Pritchard 1979b; Ligon and Lovern 2009; Moore et al. 2014).

Tishomingo National Fish Hatchery, located in southeastern Oklahoma, initiated a head-start program in 2000 and has released over 1,400 alligator snapping turtles at eight locations throughout the Mississippi River drainage since 2006. Each year during nesting season, eggs are collected from nests produced by captive brood stock and incubated until hatching. After hatching, juveniles spend one or more years in indoor enclosures to

maximize survival. The turtles are then rotated to fenced outdoor ponds until they are released at a reintroduction site. Turtles are fed daily indoors and are supplemented with extra forage when housed outdoors, conditions that were designed to result in growth that would exceed growth rates in natural populations. Surprisingly, however, the average growth rate of juvenile *M. temminckii* at Tishomingo National Fish Hatchery is generally lower than growth rates exhibited after reintroduction (Moore et al. 2013; Anthony 2015). The underlying causes of comparatively retarded growth rates in captivity are unknown.

In comparison to many sympatric freshwater turtles, *M. temminckii* is highly aquatic and exhibits a preference for benthic habitats (Sloan and Taylor 1987; Riedle et al. 2006). Hatchling, juvenile, and adult *M. temminckii* preferentially choose habitats with dense overhead canopy cover and submerged or emergent structure (e.g. flotant vegetation, submerged rocks and logs, and undercut banks) (Riedle et al. 2006; Bass 2007; Dreslik et al. 2017; Spangler 2017). Presumably, such environmental features are favored because they convey fitness advantages, such as correlating with locations of patchy dietary resources or providing protection from potential predators (Howey and Dinkelacker 2009). In addition to the immediate benefits of acquiring nutrients efficiently and avoiding violent death, these preferred conditions might offer associated benefits of reducing psychological and physiological stress and their associated impacts on fitness.

I conducted two experiments to test the effects of housing conditions and rearing densities to identify conditions that are the least stressful and most conducive for growth. My objectives were to identify captive environment conditions for juvenile *M. temminckii* that minimize stress and optimize growth, while adhering to spatial constraints that require artificial and communal housing. I tested the hypotheses that 1) increasing

structural components in housing environment and 2) reducing housing density will both result in decreased baseline corticosterone (CORT) levels and faster growth rates.

Methods

Animals and Housing Conditions. I conducted two experiments concurrently 8 June–27 July 2017 at Tishomingo National Fish Hatchery in southeastern Oklahoma. Prior to and during my experiments, all subjects were maintained in the same building under common conditions. Windows provided natural light and dictated diel light cycles. As a result, day length was comparatively long and varied throughout the experiments (mean = 14.3 h sunrise to sunset; range = 13.95–14.45 h). The building in which the turtles were housed experienced light foot traffic by hatchery staff during daylight hours, and I made no effort to eliminate this activity in order to replicate the conditions that turtles are exposed to in a typical head-start program. All of the turtles I used in my study were 21 mos old at its inception, and each turtle was double-marked using numbered/color-coded tags adhered to the carapace (Queen Marking Kits; The Bee Works, Oro-Medonte, Ontario, Canada). I only handled turtles when tanks were cleaned or when collecting blood samples and to obtain morphometrics at the beginning and end of each experiment. Rarely, individual turtles were further handled during cleaning events to replace identifying tags that had fallen off. I provided experimental animals with 6.4-mm diameter fish-based pellets three times daily at approximately 0800h, 1400h, and 2100h. I scaled the quantity of food to the number of turtles in each enclosure to limit accumulation of orts but increased quantities as the experiments progressed and turtles grew. Small quantities of orts still occurred but minimally affected water quality.

Prior approval for this project was obtained from the Missouri State University Institutional Animal Care and Use Committee (Approved: 9/2/16; IACUC ID 17-004.0).

Housing Enrichment Experiment. To test the effects of cage furnishings on growth and stress, I exposed groups of *M. temminckii* to four different housing conditions to determine their influence on growth and circulating corticosterone levels (CORT) (Appendix C). Each treatment group was characterized by the addition of one or more types of cover that correspond with preferred habitat variables that have been described from field studies (Sloan and Taylor 1987; Riedle et al. 2006; Howey and Dinkelacker 2009; Spangler 2017), while the control group was representative of the housing conditions that have historically been provided at Tishomingo National Fish Hatchery. The conditions in control tanks included placement of three segments of 15-cm diameter polyvinyl chloride (PVC) pipe (length = 30 cm), sliced longitudinally and placed with the convex side down to provide upturned edges under which turtles could find cover. Treatment 1 included the same PVC hides as the control group, with the addition of a black plastic egg crate-type light diffusers (30.5 × 39.5 cm) suspended just above the water's surface on a floating PVC tubular frame. This addition was conceived to simulate an undercut bank or floating vegetation mat, which have been identified as preferred by this species (Sloan and Taylor 1987; Spangler 2017). Treatment 2 included control conditions plus a black mesh shade cloth covering two-thirds of the top of the tank (40 cm above the water surface). Shade cloth was selected to simulate overstory canopy, which is also reportedly favored by *M. temminckii* (Sloan and Taylor 1987; Riedle et al. 2006; Shipman and Riedle 2008). Finally, treatment 3 consisted of the control group PVC

hides, the egg crate-type light diffusers, and the black mesh shade cloth over the top of the tank.

Twenty tanks (58 × 58 cm; water depth = 14 cm) were arranged in clusters of two or four (Figure 1), and 15 turtles were randomly assigned to each tank (187 cm^2 surface area per turtle). Treatments were assigned to the 20 tanks in a randomized block design with clusters serving as experimental blocks (Figure 1). After turtles were placed into their assigned tanks, they were given one week to acclimate before I collected initial blood samples and morphological measurements. These blood samples were drawn from a randomly selected subgroup of five turtles from each tank and all blood samples were collected within five minutes of handling to prevent unintended deviations from baseline CORT levels that could result from handling. I collected morphological data from every individual after blood sampling was completed. Morphometric data included mass (±0.01 g), straight midline carapace length (±0.1 mm), and plastron length (±0.1 mm). The methods described above for body measurements and blood sampling were repeated at the end of the 6-week study.

Housing Density Experiment. This experiment was designed to assess the potential influence of housing density on *M. temminckii* growth and CORT levels. I housed turtles in opaque plastic containers (43 × 30 cm) (Traex Corp.; Dane, WI, USA) that were randomly positioned on two levels of stacked shelving (Figure 2). Each container contained two PVC hides (15-cm diameter PVC; length = 30 cm) placed convex-side down. A total of 24 containers (12 per shelf) were used to obtain eight replicates for each density treatment. I randomly reassigned the positions of containers within and across the two shelving levels weekly to eliminate potentially confounding

effects of microclimate variation within the building. Three experimental groups were designated: (1) turtles housed solitarily (1,290 cm^2 per turtle); (2) turtles housed in medium-density conditions of five individuals per container (258 cm^2 per turtle); and (3) turtles housed in high-density conditions of 10 individuals per container (129 cm^2 per turtle). The same growth measurements and blood sampling were performed at the inception and end of the experiment as described in the housing enrichment experiment. I drew blood samples from one representative turtle from each enclosure, followed by collection of morphometrics from every turtle.

Blood Sampling and Corticosterone Radioimmunoassay. I collected blood samples (0.2–1.0 mL) from animals in both experiments at the beginning and end of each experimental trial. The same animals were sampled at each interval to facilitate analyzing data in a repeated measures framework. Samples were taken from the caudal artery on the dorsal side of the tail with a 21-gauge needle and 1-mL syringe (Teare 2010). Blood samples were collected within five minutes of a turtle being removed from its tank, which is predicted to be representative of its baseline circulating CORT concentrations (Polich 2016; D. Thompson, pers. comm.). Samples were initially stored on ice and later centrifuged. I then transferred plasma to new storage tubes and froze them at -20 °C. Finally, corticosterone concentrations were determined via radioimmunoassay using 100 µL aliquants and following methods described in Love et al. (2017).

Statistical Analysis: Housing Enrichment. Results from the housing enrichment experiment were analyzed in a randomized design to compensate for potentially heterogeneous environmental conditions within the building in which the study was conducted. I treated tanks as random factors, treatment groups as fixed factors, and

individual turtles were experimental units. I checked the distributions of initial body size, CORT, and growth rate data for normality using Shapiro-Wilk tests, and I used non-parametric Kruskal-Wallis tests when data failed the assumption of normality. Prior to beginning the experiment, I ensured that variance of body size was equal among treatments using a Shapiro-Wilk test. To determine the influence of housing enrichment treatments on growth, I analyzed size-corrected growth rates to adjust for variation in body size at the inception of experiments, with rates expressed in $mg \cdot g^{-1} \cdot d^{-1}$. I compared turtles' growth rates over the duration of the experiment across treatments using a non-parametric Kruskal-Wallis test. I tested for effects of end-of-trial CORT levels on growth rates using linear regressions. To compare growth rates with experimental groups and tanks as main effects, and baseline CORT level changes between beginning and end of the experiments as the response variable, I used a two-factor repeated-measures analysis of variance (ANOVA). I analyzed differences in baseline CORT samples among blocks, treatment groups, tanks, and period (beginning versus end samples) by using a multiple-factor repeated-measures ANOVA.

Statistical Analysis: Housing Density. The housing density experiment was analyzed using a completely randomized design, with treatment as a fixed factor and tanks representing experimental units. This design was necessary because the low-density tanks contained just one turtle each and therefore within-tank variation could not occur. As described for my housing experiment, I calculated size-corrected growth rates ($mg \cdot g^{-1} \cdot d^{-1}$); I also used Kruskal-Wallis non-parametric tests when initial body size, growth rate, or CORT concentrations failed the assumption of normality. I began by testing for the effect of housing density on growth rates over the duration of the experiment using a

Kruskal-Wallis test. I then conducted one-factor ANOVAs to assess the effects of housing density on the mean, minimum, and maximum growth rates of turtles in each tank. By necessity, all three values were represented by the same individuals in the low-density treatment. I used linear regression to examine the effects of end-of-trial CORT levels on growth rates. Using a repeated-measures ANOVA, I was able to compare CORT level values among periods and experimental groups. Finally, I used a repeated-measures ANOVA to compare growth rates with treatments and periods as main effects, and the response variable as baseline CORT level change over the duration of the experiment.

Results

Housing Enrichment. Three hundred turtles were initially assigned to treatments in the housing enrichment experiment. However, four animals' blood samples were omitted from CORT assays either because plasma sample volumes were insufficient or else were contaminated with lymph. Additionally, one turtle died from injuries inflicted by a cage mate. Notably, overall aggression was exceedingly rare and was only observed when animals were disturbed during cage cleaning. The deceased turtle was replaced with another turtle of the same age class to maintain consistent housing density; however, data from these two individuals were omitted from analyses.

At the beginning of this experiment, morphometrics of the 21-month-old alligator snapping turtles were equal among treatments and averaged 102.0 ± 1.41 g ($H = 0.161$, $p = 0.98$), 69.75 ± 0.33 mm straight mid-line carapace length ($F_{3,295} = 0.190$, $p = 0.90$), and 57.34 ± 0.28 mm plastron length ($F_{3,295} = 0.114$, $p = 0.952$). Turtles grew an average of

18.24 ± 0.35 mg·g^{-1}·d^{-1} (range = 4.04–36.34 mg·g^{-1}·d^{-1}) over the course of the 6-week trial; however, growth rates were strongly influenced by housing enrichment treatment, with turtles in the two treatments that contained flotant structures growing much faster than either the control or the treatment with shade-cloth canopy ($H = 31.24$, $p < 0.0001$; Figure 3). Among the subset of 76 turtles from which useable blood samples were obtained, growth rate did not correlate with plasma CORT levels measured at the end of the experiment ($r^2 = -0.0031$, $p = 0.59$; Figure 4A). Plasma CORT was not affected by treatment, and there was no interaction between sampling period and treatment (treatment: $F_{3,184} = 0.78$, $p = 0.51$, period × treatment: $F_{3,184} = 0.10$, $p = 0.96$). However, there was a strong and consistent effect of sampling period on CORT ($F_{1,184} = 8.90$, $p = 0.0032$; Figure 5A).

Housing Density. One hundred twenty-eight turtles were distributed across 24 enclosures comprising three levels of density. Mean growth rates across treatments was 21.70 ± 0.44 mg·g^{-1}·day^{-1} (range = 8.17–32.60 mg·g^{-1}·day^{-1}) and was not significantly influenced by stocking density ($H = 0.09$, $p = 0.96$). However, there were significant differences when the turtles housed singly in the low-density treatments were compared to the fastest and slowest-growing turtles in the medium- and high-density treatments, ($F = 10.501$, $df = 2$ $p < 0.001$; Figure 6). Specifically, the fastest-growing turtles maintained in high-density ($p < 0.001$) and medium-density ($p = 0.013$) conditions grew faster than those in the low-density groups. However, there were no differences observed between fastest-growing animals in the high-density and medium-density groups. Conversely, the slowest-growing turtles in the low-density group grew faster than those in the high-density treatment ($p < 0.05$). I did not observe any differences between slowest-growing

turtles in the low- and medium-density treatments ($p > 0.05$) or the medium- and high-density treatments ($p > 0.05$)

In contrast to results from the housing enrichment experiment, I observed a negative correlation between CORT at the end of the experiment and growth (slope = -1.99, $r^2 = 0.40$, $p < 0.001$; Figure 4B). Neither housing density nor interaction between housing density and period had a significant effect on average baseline CORT (treatment: $F_{2,21} = 1.73$, $p = 0.20$, period × treatment interaction: $F_{2,21} = 0.23$, $p = 0.80$). However, CORT was consistently lower at the end of the experiment than at the beginning ($F_{1,21} = 4.58$, $p = 0.0443$; Figure 5B).

Discussion

Field studies of adult, subadult, and hatchling alligator snapping turtles have consistently reported associations with overstory canopy, as well as various aquatic structures that have included woody debris, rocks, undercut banks, and mats of floating aquatic plants (Harrel et al. 1996; Riedle et al. 2006; Bass 2007; Moore et al. 2014; Spangler 2017). When organisms associate with habitat features at frequencies that are greater than would occur randomly, it is typically assumed that there is a fitness benefit to doing so. Interestingly, while my housing enrichment experiment sought to simulate these habitat characteristics, growth rates of juvenile alligator snapping turtles were only greater in enclosures containing floating mats that mimicked flotant vegetation and were unaffected by the presence of a shade cloth implemented to mimic overstory canopy. Thus, the likely fitness benefits of floating mats—and hence the preference for it in nature—are reasonably clear, whereas associating with dense overstory canopy either

enhances fitness in some way other than increasing growth rates or else simply does not convey a fitness advantage. While many studies have reported preferences for these and other habitat features, to my knowledge no study has attempted to rank the relative importance of preferred environmental variables. Alternatively, it is possible that shade cloth covering the tops of tanks was simply not perceived in the same way by captive turtles as overstory canopy is by wild turtles. Finally, it is possible that alligator snapping turtles do not in fact exhibit a preference for overstory canopy, but rather are found in association with it due to some highly correlated variable, such as water depth, that was not represented in my experiments.

Throughout my housing enrichment experiment, turtles were typically closely grouped below the flotant structure and were rarely seen in close proximity to PVC segments beyond the perimeter of the mat (Appendix D). The surface area of the flotant afforded refuge for every animal, which may have been conducive to reducing competition within tanks (Oonincx and Leeuwen 2017). In contrast, only a subset of animals could be in close proximity to sides of PVC segments, leaving others exposed and likely to jockey for a preferred position in the tank. Higher growth rates of *M. temminckii* maintained in tanks with flotant structures may be due to reduced energy expended on activity resulting from reduced competition for the limited cover provided by PVC segments, or could have resulted from increased food consumption. Although I did not quantify either variable, there appeared to be consistently shorter latency to feed and less orts in tanks that were afforded cover by a flotant mat. This may be due to the turtles' perception that they were undetectable under the mats.

My hypothesis that growth rates would inversely correlate with housing density was not supported, as housing density did not affect average growth across density groups. However, as turtle density increased, so too did the average mass gained by fastest-growing turtle in each tank. The inverse relationship was apparent for slowest-growing turtles, which grew more slowly at both medium and high densities than turtles that were housed singly. These patterns clearly indicate high variability in growth rates, even as the average collective growth rate remained consistent among groups. I interpret these patterns to indicate that communal housing in turtles results in a distinct division of winners and losers of resources. Food was allocated based upon the number of turtles in an enclosure, and presumably turtles that fed immediately or otherwise actively competed for food were able to co-opt disproportionate quantities. Meanwhile, turtles that were reluctant to eat obtained less food. These patterns suggest the possibility of dominance hierarchies in groups of alligator snapping turtles, at least in a captive setting. In support of this possibility, subsets of individuals in medium- and high-density groups typically displayed immediate feeding responses, and all or most food presented to them was consumed prior to the next feeding event. While feeding, turtles in these groups would frequently mount other turtles to obtain food, stretch their necks past other individuals, or forcefully move other turtles to eat. These behaviors closely resembled those reported for common snapping turtles housed communally, which resulted in differential food acquisition (Froese and Burghardt 1974). Grimpe (1987) noted that two captive adult *M. temminckii* exhibited a hierarchical relationship characterized by a male aggressor that frequently bit a submissive female cage mate, as well as the females' retreat from food when the dominant male approached. Reduced body condition of wild male *M.*

temminckii in the presence of well-nourished females has also generated speculation for male-male competition in response to population growth (Trauth et al. 2016). The fact that I observed similar patterns among small juveniles that were similar in size suggests that dominance hierarchies may not result solely from variation in size or male dominance.

A study of hatchling common snapping turtles (*Chelydra serpentina*) found that growth rates were higher when animals were housed in isolation, rather than in groups (McKnight and Gutzke 1993). This contrasted with my results in which average growth rates of turtles maintained solitarily were not significantly different from those housed communally. Solitary turtles were also rarely seen eating and often left orts. I speculate that the lack of competition resulted in a weak feeding response. Based on the understudied behavioral ecology of *M. temminckii* in the wild, it is possible that some level of competition with conspecifics or guild mates may be beneficial. My study was not designed to formally investigate behavior, but similar experiments could be conducted to resolve the role of competition in triggering feeding activity and, by extension, growth.

Contrary to my prediction that high group density and low housing enrichment would trigger a stress response in juvenile alligator snapping turtles, average CORT levels did not vary among treatments in either experiment. There are two potential causes for this outcome: 1) the turtles were equally physiologically stressed (or not) across treatment groups in both experiments, or 2) corticosterone is not a reliable indicator of stress in this taxon. Although plasma CORT is considered the dominant glucocorticoid in reptiles (Sandor and Mehdi 1979; Jessop 2005; Wada 2008; Silvestre 2014), it is possible

that some reptile taxa deviate from this broad phylogenetic pattern. My study is the first that I am aware of to report stress hormone levels of alligator snapping turtles, and it is conceivable that corticosterone is less active than cortisol. Indeed, the physiological activity of cortisol in a physiological stress response has been demonstrated in studies of Asian soft-shelled turtles (*Pelodiscus sinensis*) (Zhou et al. 2003) and Hermann's tortoises (*Testudo hermann*i) (Fazio et al. 2014). To my knowledge, no study to date has compared the relative activity of different glucocorticoids in the stress response of turtles, although among non-avian reptiles at least one lizard has been demonstrated to physiologically respond to both corticosterone and cortisol (Jacob and Oommen 1992).

Alternative explanations for the lack of treatment effects on CORT levels are that animals were either equally unstressed across treatments (concentrations were generally low), or that CORT is simply a poor indicator of stress in some taxa or under some conditions. Heterophil to lymphocyte ratios (H/L) have been shown to complement CORT levels in turtles and have even been suggested as a more dependable index for chronic stress (Aguirre et al. 1995; Morici et al. 1997; Adamovicz et al 2015; Polich 2016). For example, eastern box turtles (*Terrapene carolina carolina*) had lower H/L ratios but displayed no difference in fecal CORT or growth when provided with an enriched environment (Case et al. 2005). Other potential indicators of chronic stress should be investigated, such as stress-induced CORT and cortisol, immune response, respiration rate, complete hemogram and differential analysis, and oxidative damage (Aguirre et al. 1995; Romero and Wilkelski 2001; Selman et al. 2012).

The effect of period on CORT levels in both experiments may be a consequence of insufficient acclimation time prior to initiating the experiments and/or the influence of

housing conditions prior to the start of the experiment. Turtles were relocated from large raceway tanks to smaller tanks of similar densities. Moving reptiles to smaller areas of habitat can cause a heightened influence or change of hierarchy in close quarters, leading to a period of adjustment for social stability (Greenberg 1987). Baseline CORT levels of wild *M. temminckii* should be measured to compare my results to concentrations typical for the species. Additionally, experiments testing the acute response of alligator snapping turtle circulating CORT to stress would be helpful for assessing its role in the stress response.

The relationship between CORT and growth supported my predictions in one experiment but did not in the other. The lack of a CORT/growth relationship in the housing enrichment experiment suggests that CORT either does not influence growth in a predictable way or that CORT is a poor indicator of stress in alligator snapping turtles. However, the expected negative relationship I observed in the housing density experiment, with high CORT levels corresponding with low growth rates suggest that, at least under some conditions, elevated CORT does in fact suppress growth. Plasma CORT concentrations were similar in the two experiments, so it seems unlikely that one set of treatments triggered a stress response and the other did not.

Implications for Captive Propagation. My results demonstrate the importance of how captive environmental enrichment can maximize growth of juvenile alligator snapping turtles for reintroduction. Additions to enclosures that emulate structures that turtles use in nature may be useful, but clearly some types of enrichment produce measurable benefits to individual fitness whereas others do not. The effects of housing density are less clear and the 'right' density may be context-dependent. Group housing

produced variable growth rates within cages, suggesting that there are winners and losers under such conditions. While there is still room for debate for the effectiveness of head-starting as a conservation tool, the high mortality rates that young *M. temminckii* sustain before reaching sizes that exceed predator gape limits are still problematic for restoring wild populations (Dreslik et al. 2017). This suggests that there is in fact value to head-starting alligator snapping turtles, and that experimentally evaluating different rearing conditions can identify optimal husbandry methodologies that produce turtles that are optimally conditioned prior to release.

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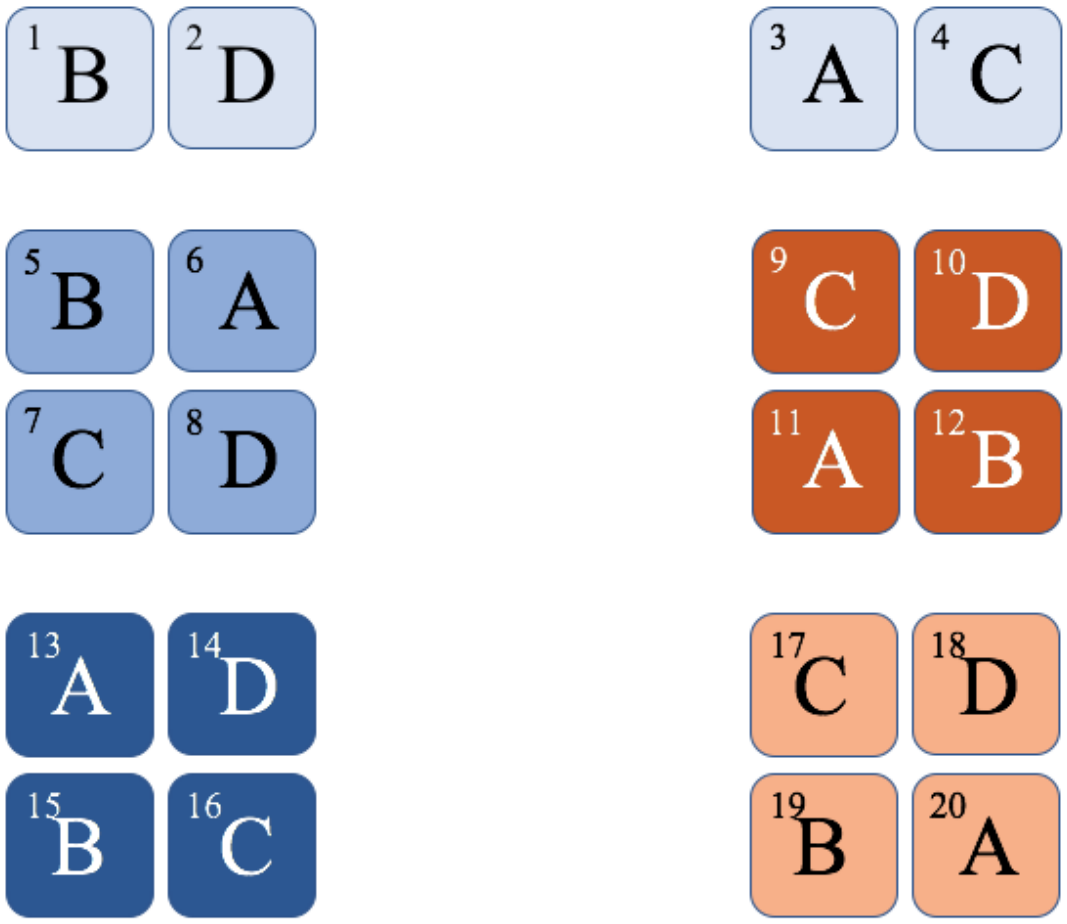

Figure 1. Aerial view of the juvenile *M. temminckii* tank arrangement in the housing enrichment experiment. Each color represents one of five experimental blocks. Letters indicate the housing treatment experimental unit in a randomized block design.

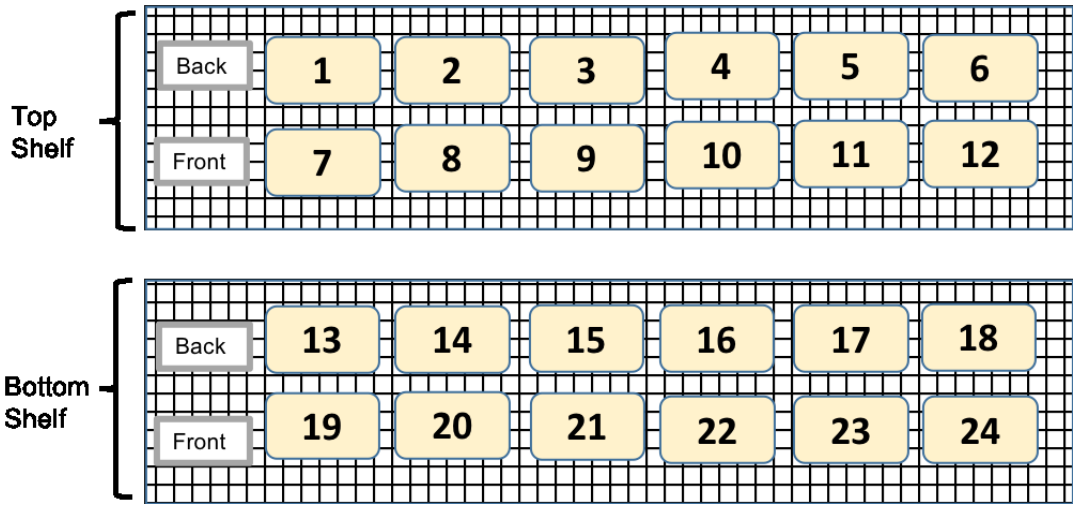

Figure 2. *M. temminckii* enclosures divided into three treatments groups of varying densities spanning across two webbed plastic shelves. Containers were rotated weekly to account for potential confounding variables in the environment.

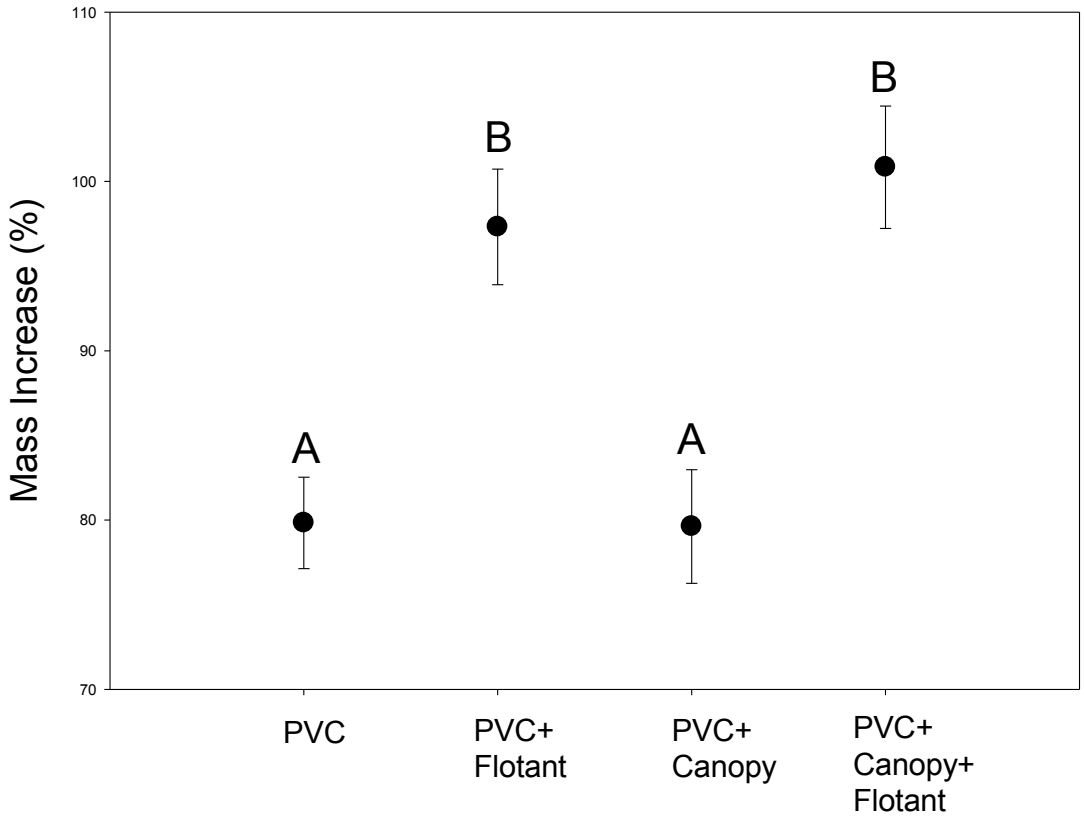

Figure 3. The percent change in mass of juvenile *M. temminckii* housed in tanks containing four combinations of structural enrichment components. Error bars are ± 1 SE.

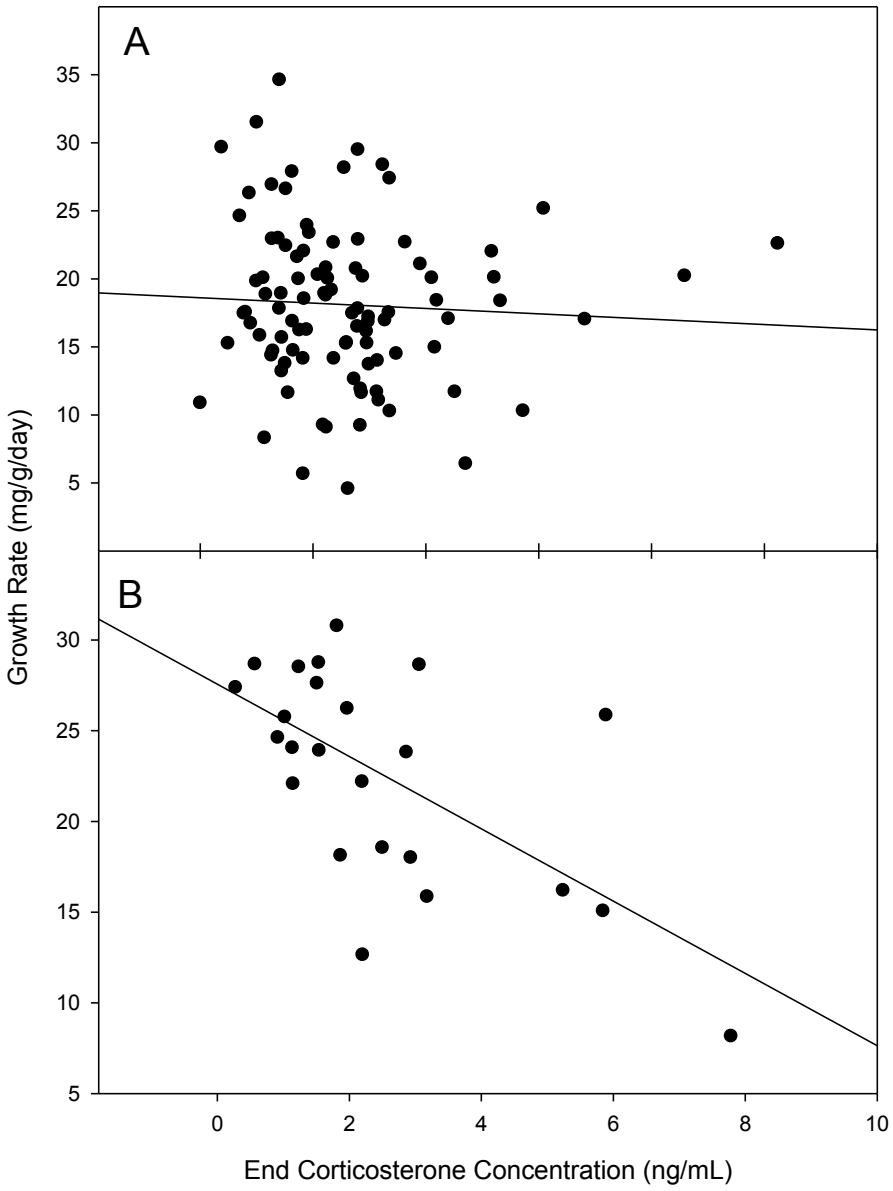

Figure 4. The relationship of *M. temminckii* baseline corticosterone levels to growth rates at the end of two captive housing experiments, which manipulated A) housing structural enrichment ($r^2 = 0.003$, $p = 0.59$) and B) stocking density ($r^2 = 0.40$, $p < 0.001$, slope = -1.99)

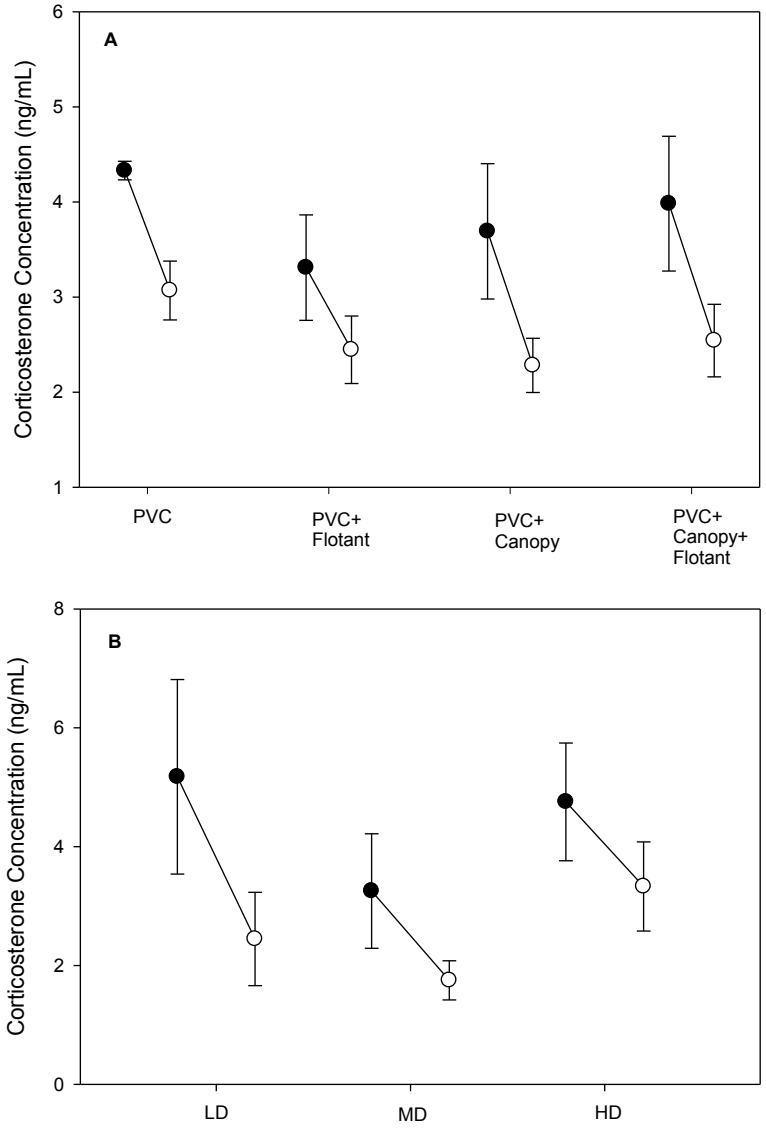

Figure 5. Average baseline CORT of a subset of juvenile *M. temminckii* sampled at the beginning and end of two 6-week experiments. A) Turtles were maintained in enclosures varying in structural enrichment types. B) Turtles were housed in low-density, medium-density, and high-density conditions. Closed circles = CORT at start of density experiment, open circles = CORT at end of density experiment. Error bars are ± 1 SE.

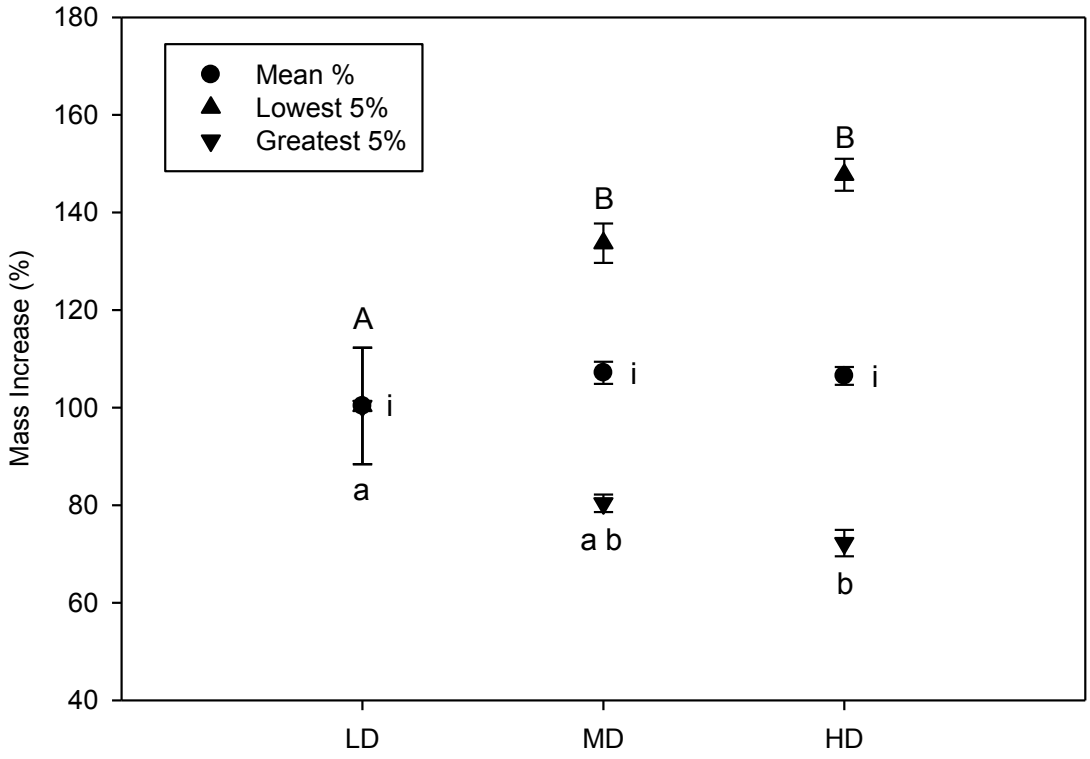

Figure 6. The percent change in mass (g) displayed by alligator snapping turtles housed in low-density, medium-density, and high-density conditions. Mean percentage change of mass, and the fastest- and slowest-growing individuals over the course of the study are represented. Roman numerals, uppercase letters, and lowercase letters illustrate the relationships among treatments for comparisons of the mean, maximum, and minimum growth rates, respectively. Error bars are ± 1 SE.

SUMMARY

By reviewing the growing base of literature on the life-history, ecology, and behavior of the alligator snapping turtle (*Macrochelys temminckii*) and closely-related species, I was able to simulate a few of the elements that may benefit this species in the wild. The importance of optimizing growth and fitness of juvenile alligator snapping turtles in a head-start program extends far beyond the immediate successes of releasing a healthy cohort into the wild. Elevated rates of juvenile growth and development reduces risks of depredation, increases the animal's ability to compete for resources, and could reduce the amount of time before reaching sexual maturity.

Juvenile turtles provided with one or a combination of tank furnishings including submerged PVC pieces, floating mats, and black mesh shade cloth canopy only showed elevated growth rates in treatments containing floating mats. Stress did not appear to be the influencing factor of growth rates in my housing experiment, but further experimentation of stress response in this species is warranted. The density at which the turtles were housed did not have an effect on average growth rates, but higher densities did create a larger divide in growth between the largest and smallest turtles in each enclosure. Behavioral observations during the experiment suggest that hierarchical competition may be the source of this divide.

Hatchlings inoculated with adult conspecific feces after hatching were better at assimilating fiber than turtles inoculated with creek water and deionized water. Growth was not significantly different across treatments, but hatchlings receiving adult feces consistently maintained larger body masses each week, which seemed to become more prominent toward the end of the 6-week experiment. Core microbiomes were consistent

across hatchling groups. Notable changes were observed between the first fecal sample and the final fecal sample in each treatment, suggesting that the turtles' microbiomes change over time based on differential exposure to microbes.

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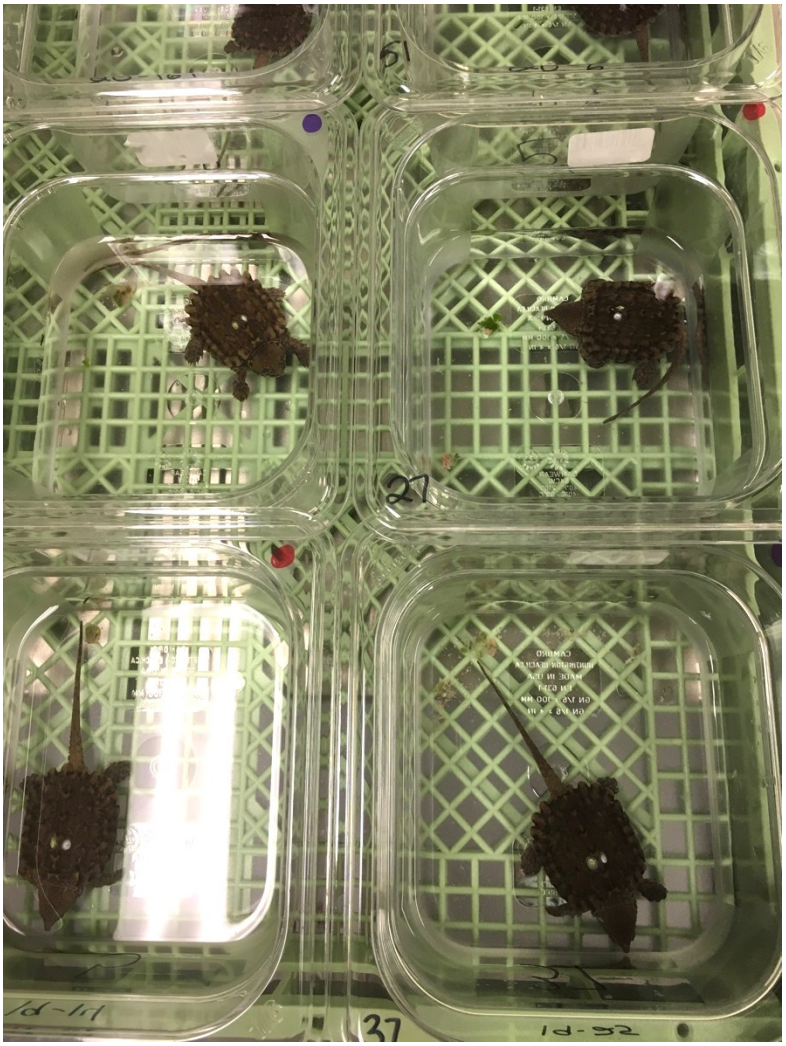
APPENDICES

Appendix A

Hatchlings in the digestive efficiency experiment housed singly in 1.9-L polycarbonate containers filled with 400 mL of deionized water. Containers were labeled with a unique number matching its turtle's unique tag number, along with its treatment color code.

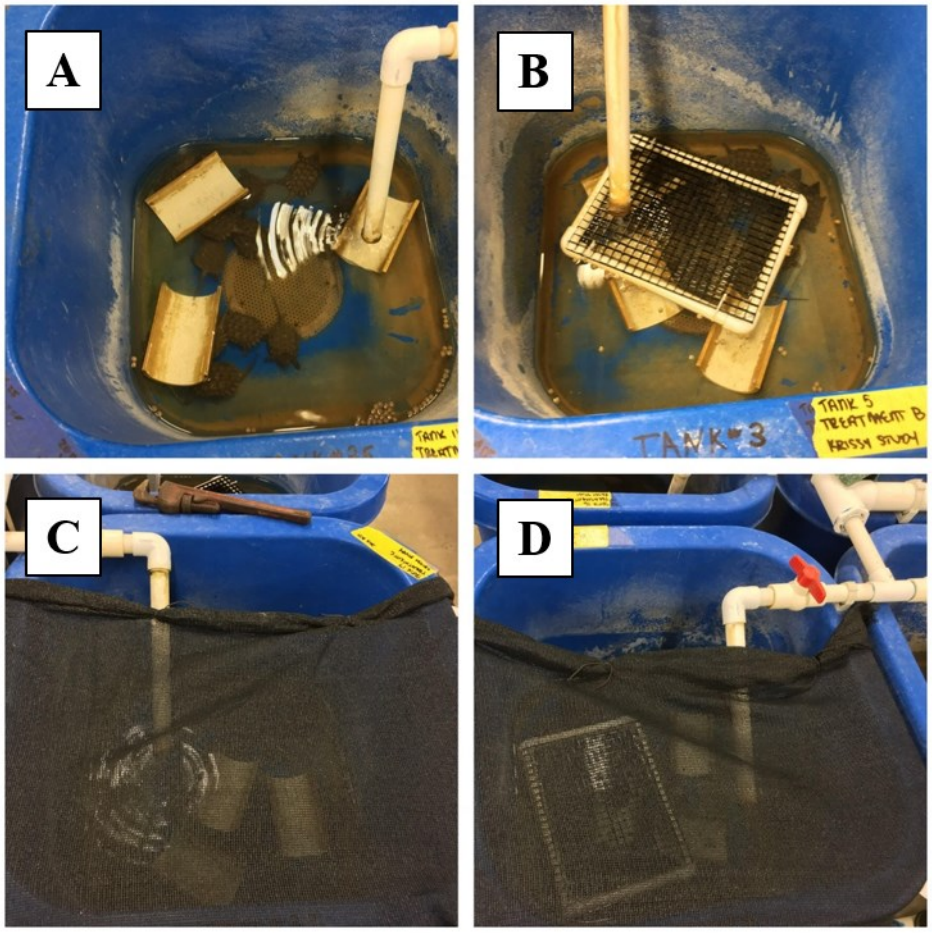


Appendix B

Hatchlings housed across two environmental chambers maintained at 28 °C, with a 12-hour light cycle. Each hatchling container was rotated with their individual 1.9-L containers throughout the chambers to reduce the potential confounding effects of microclimate.

Appendix C

The four treatment groups of the housing enrichment experiment at Tishomingo National Fish Hatchery were characterized by A) three PVC segments cut longitudinally and placed convex-side down, B) PVC segments and a floating mat with PVC frame, C) PVC segments and a black mesh shade cloth covering two-thirds of the tank, and D) all three structural components.



Appendix D

Juvenile *M. temminckii* congregating under a floating PVC mat at Tishomingo National Fish Hatchery that was used in the housing enrichment experiment.