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Assessment of Blood Lead, Zinc, and Mercury Concentrations and Cholinesterase Activity in Captive-reared Alligator Snapping Turtles (*Macrochelys temminckii*) in Louisiana, USA

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ABSTRACT: The alligator snapping turtle (*Macrochelys temminckii*) is a freshwater apex predator that has experienced severe population declines throughout its range due to historical overharvesting and habitat degradation. Because of its long lifespan, high trophic level, and limited home range, it is a suitable sentinel species for monitoring environmental contaminants. In Louisiana, US a pilot program aims to augment free-ranging populations by releasing captive-reared individuals. Baseline values of potential environmental contaminants were determined as part of an overall health assessment to evaluate captive-reared alligator snapping turtles for release. Blood samples from 3-yr-old (n=23) and 4-yr-old (n=11) captive-reared alligator snapping turtles were tested for lead (Pb), mercury (Hg), and zinc (Zn) levels by atomic absorption spectrophotometry and cholinesterase (ChE) activity (as a biomarker for organophosphate and carbamate exposure) by the modified Ellman method. Reference intervals were determined for Zn (34 to 295 µg/dL), Hg (0 to 4.8 µg/dL), and ChE (0.17 to 1.65 µmole acetylthiocholine/mL per minute). Elevations of Pb, Zn, or Hg, or decreases in ChE activity levels of this cohort during recapture sampling may indicate point-source intoxications or bioaccumulation, both ultimately attributable to environmental contamination. The released animals may serve as sentinels for biomonitoring of their new habitat for the evaluated toxicants.

Key words: Biomonitoring, contaminants, ecosystem health, heavy metals, sentinel.

The alligator snapping turtle (*Macrochelys temminckii*) is endemic to North American watersheds draining to the Gulf of Mexico and has suffered severe population declines throughout its range due to historical overharvesting (Boundy and Kennedy 2006). Commercial harvest has been universally banned, although limited harvest for personal consumption remains legal. Long life spans and a high trophic level, combined with small home ranges, low dispersal rates, and fidelity to home waterways, make this aquatic carnivore an ideal sentinel for monitoring the health of freshwater ecosystems (Chaffin et al. 2008).

Blood sampling is a practical, minimally invasive, nonlethal approach used under field conditions to conduct health evaluations (e.g., clinical and molecular diagnostics) and to monitor concentrations of essential (e.g., zinc) and nonessential (e.g., lead, mercury) metals in free-ranging chelonians (Golet and Haines 2001; Hopkins et al. 2013a; Ehsanpour et al. 2014). In vertebrates, erythrocytes and plasma contain cholinesterase (ChE), an enzyme inhibited by organophosphates (OP) and carbamates (CB), agrochemicals widely used as pesticides. Blood ChE activity has been used as a biomarker for OP and CB exposure in free-ranging reptiles (Sanchez-Hernandez and Walker 2000). We assessed blood lead (Pb), mercury (Hg), and zinc (Zn) concentrations and ChE activity in a population of captive-reared alligator snapping turtles prior to their release to free-range.

Hatchlings from a commercial turtle farm in Monroe, Louisiana, US, were raised by the Louisiana Department of Wildlife and Fisheries for 1 yr indoors in temperature and photoperiod-controlled raceways before transfer to artificial outdoor ponds for an additional

2–3 yr prior to release. Breeding stock included captive-hatched and wild-caught animals, although all original founders were wild-caught in Louisiana. Three- (n=23) and 4-yr-old (n=11) turtles were housed in separate ponds filled with water from the adjacent bayou, introducing natural prey such as catfish (*Ictalurus* spp.), crappie (*Pomoxis* spp.), and crayfish (*Procambarus* spp.) and were stocked with native bluegill (*Lepomis* spp.) to provide live forage. Diets were supplemented with commercially purchased raw whole chicken and American shad (*Alosa sapidissima*) each spring, increasing to a peak frequency of 2–3 times per week in summer and tapering in autumn. Diets were not supplemented in winter.

In August 2015, turtles were captured by net over 3 wk and moved to indoor raceways for physical examination and venipuncture. Each turtle was identified by a metal tag in a carapacial marginal scute and an intramuscular passive integrated transponder (FriendChip, Avid Identification Systems, Norco, California, USA). Approximately 3 mL whole blood were drawn via a 22-ga needle from the dorsal coccygeal vein of each turtle and immediately transferred to plastic lithium heparin tubes (Microtainer™ Blood Collection Tube, Becton, Dickinson, Franklin Lakes, New Jersey, USA) and stored in a cooler with ice for approximately 8 h before refrigeration at 5 C for 24 h prior to analysis.

Total Pb and Hg were measured in whole blood by graphite furnace atomic absorption spectrophotometry (AAS) using a Zeeman background correction (PE Analyst 800, PerkinElmer, Norwalk, Connecticut, USA) with a limit of detection (LOD) of 0.4 μg/dL and by cold vapor AAS (Flow Injection Mercury System 400, PerkinElmer; LOD=0.5 μg/dL), respectively. For both assays, heparinized whole blood was diluted 11-fold and hemolyzed with 0.01% Triton X-100 (Fisher Scientific, Pittsburg, Pennsylvania, USA). Seronorm™ Whole Blood Level 2 (Sero, Billingstad, Norway) was used as a quality assurance control in both assays.

Zinc was measured in plasma by AAS (PE 5000, PerkinElmer) with a deuterium-tungsten background correction (LOD=2 μg/dL). Plasma was diluted sufficiently to compare with aqueous standard solutions. Standard bovine nonfat milk powder (National Institute of Standards and Technology, US Department of Commerce, Gaithersburg, Maryland, USA) was used as a quality assurance control.

Cholinesterase activity was determined by the modified Ellman method (Ellman et al. 1961). Heparinized blood was hemolyzed, diluted as with Pb and Hg assays, and further diluted 40-fold with 0.05 M Trizma buffer, pH 7.4 (Sigma Diagnostics, St. Louis, Missouri, USA). Two milliliters of this dilution were mixed with 1 mL 0.5 mM dithiobis(2-nitrobenzoic acid) (Sigma Diagnostics) chromogenic reagent and 0.1 mL acetylthiocholine (Sigma Diagnostics) in a polystyrene cuvette (Scientific Commodities, Lake Havasu City, Arizona, USA). Change in absorbance was measured by spectrophotometry (Shimadzu UV 1800, Shimadzu Corporation, Kyoto, Japan) at 412 nm at 30-s intervals for 90 s. Accutrol serum (Sigma Diagnostics) was used as a quality assurance control.

Distribution of the data for each variable was plotted on histograms and the normality of distributions tested with the D'Agostino-Pearson test. Extreme outliers were identified with the Tukey procedure and excluded from reference interval (RI) estimation. Reference intervals were calculated with the robust method, and 90% confidence intervals for upper and lower intervals were estimated (Friedrichs et al. 2012). Linear correlations between numeric variables (Hg and Zn, Hg and ChE, Zn and ChE) were tested with Pearson's or Spearman's rank correlation, as indicated by the distribution of the variable. Association between variables and age group was calculated by the Mann-Whitney U-test or Student's t-test, as indicated by the distribution of the variable. Analysis was performed with commercial software (SPSS version 22, IBM, Armonk, New York, USA). Statistical significance was defined as two-tailed, $P \leq 0.05$.

No extreme outliers were detected for Zn, Hg, or ChE. Four extreme outliers were detected for Pb (9, 6, 3, and 3 μg/dL) and

excluded. All Pb values were ≤2 μg/dL; therefore, RIs were not calculated. Calculated RIs were wider than the range of measured values, which is not unexpected when robust methods are employed (Horn et al. 1998). Reference intervals were determined for Zn, Hg, and ChE (Table 1). Blood trace metal concentrations and ChE activity were evaluated for both 3- and 4-yr-old turtles (Table 2). No significant differences were found for Pb, Zn, or ChE between the 3- and 4-yr-old turtles, but Hg was higher in the 4-yr-old than in the 3-yr-old cohort (Mann-Whitney *U*-test: $P=0.029$).

Blood Pb concentrations were below the LOD for most individuals, consistent with surveys of free-ranging alligator snapping turtles (Chaffin et al. 2008). Lead is found in erythrocytes and stored in tissues and keratinized structures where it is biologically unavailable. Therefore, blood Pb concentrations reflect recent ingestion or redistribution from tissue such as bone (Grillitsch and Schiesari 2010). This may be important during osteoclastic activity, such as that associated with reproductively active females, which may cause blood Pb to increase. To assess for Pb exposure, scute or nail samples could be collected during resampling efforts as a minimally invasive diagnostic alternative to blood (Grillitsch and Schiesari 2010).

An essential element, Zn is bound to plasma proteins in blood and is normally found in all tissues (Ehsanpour et al. 2014). Blood Zn concentrations in this population of captive-reared alligator snapping turtles were lower than those reported for free-ranging counterparts (Chaffin et al. 2008), potentially due to differences in dietary Zn content or to the smaller sizes of the captive-reared individuals, considering blood Zn increases with increasing body mass in other chelonians (Ehsanpour et al. 2014). Because the toxic threshold of Zn in alligator snapping turtles is unknown, establishing RI for age cohorts will help to provide baselines to aid in future diagnostic evaluation.

Blood Hg concentrations correlate with muscle concentrations in other chelonians (Golet and Haines 2001; Day et al. 2005), making blood Hg a good indicator of potential human exposure through consumption of turtle meat. Total Hg blood concentrations of this population were below the consumption threshold of 100 μg/dL recommended by the US Environmental Protection Agency and the US Federal Drug Administration (Green et al. 2010). Due to bioaccumulation, Hg concentrations in organs or keratinized tissues are more-accurate reflections of Hg exposure over time (Hopkins et al. 2013a). In other chelonians, correlation of blood Hg concentrations with tissue Hg concentrations and with body mass suggest blood Hg concentrations do not only reflect recent dietary exposure (Day et al. 2005; Hopkins et al. 2013b). This may explain higher blood Hg concentrations in 4-yr-old versus 3-yr-old turtles. Blood Hg concentrations in our captive-reared population were much lower than those reported from free-ranging alligator snapping turtles, which generally exceeded 10 μg/dL (Chaffin et al. 2008). This may be because this population had less Hg exposure or because it was comprised of younger, smaller animals.

The RI for baseline ChE activity for our captive-reared population was higher than that reported for free-ranging alligator snapping turtles (Chaffin et al. 2008), potentially because this population experienced less exposure to OP and CB, having been raised under captive conditions not associated with an agricultural landscape. Alternatively, ChE activity may vary in alligator snapping turtles, as it does in other species, by age and sex or according to season or time of day of sample collection and the methodology employed (Sanchez-Hernandez and Walker 2000; Fildes et al. 2009). Organophosphate and CB exposure will decrease ChE activity in blood before affecting the central nervous system. Therefore, blood ChE activity may be used to obtain an antemortem diagnosis of acute OP or CB exposure, although brain samples may be more appropriate for postmortem diagnosis of OP or CB intoxication.

It is assumed the outdoor habitat and naturally occurring prey were primary sources of exposure of the captive-reared population

TABLE 1. Reference intervals (RI) for blood lead (Pb), zinc (Zn) and mercury (Hg) concentrations and cholinesterase (ChE) activity calculated by the robust method (with 90% confidence interval [CI]) for juvenile alligator snapping turtles (*Macrochelys temminckii*; *n*=34) captive-reared in Louisiana, USA.[a]

Variable	Mean	SD	Range	Lower RI	90% CI for lower RI[b]	Upper RI	90% CI for upper RI[b]
Pb (μg/dL)[c]	—	—	≤2–9[d]	—	—	—	—
Zn (μg/dL)	170	60	60–350	34	5–70	295	257–328
Hg (μg/dL)	2	1	1–5	0	—	4.8	—
ChE (μM ASCh/mL per minute)	0.98	0.35	0.34–1.76	0.17	0.01–0.35	1.65	1.45–1.88

[a] — = not calculated; ASCh = acetylthiocholine.

[b] Calculation of 90% CI for the RI was not possible when the sample contained too many equal values.

[c] Calculation of mean was not possible because most values fell below the lower limit of detection (Pb ≤2 μg/dL).

[d] Range includes outliers; if outliers were excluded, all values of Pb were ≤2 μg/dL.

to environmental contaminants and mimic those of free-ranging counterparts. Supplemental feed was commercially sold for human consumption and is unlikely to have contributed to contaminant exposure. The potential effect of maternal transfer of metals (Ehsanpour et al. 2014), OP, and CB (García-Besné et al. 2015) on the captive-reared population is unknown. Future efforts should include serial monitoring of water, sediments, and food items as well as testing of female breeding stock.

Assessment of trace elements is an integral part of health surveillance of animal populations (Jin et al. 2015), but the significance of findings is often hindered by unknown baselines. Having established baseline blood Pb, Zn, and Hg concentrations and ChE activity, future resampling of this population may provide the opportunity to study these parameters over time. Deviations from baseline may suggest point-source intoxication or bioaccumulation, both ultimately attributable to environmental contamination. Due to a limited sample size, the RIs that we determined for Zn and Hg concentrations and ChE activity may be appropriate baselines for this population but should be used with caution for comparison with other animals.

TABLE 2. Median and SD blood zinc (Zn) and mercury (Hg) concentrations and cholinesterase (ChE) activity for 3-yr-old (*n*=23) and 4-yr-old (*n*=11) cohorts of juvenile alligator snapping turtles (*Macrochelys temminckii*) captive-reared in Louisiana, USA.

	3-yr-old turtles		4-yr-old turtles	
Variable	Median	SD	Median	SD
Zn (μg/dL)	190	50	150	67
Hg (μg/dL)[a]	2	1	3	2
ChE (μM ASCh/mL per minute)[b]	1.04	0.35	0.8	0.35

[a] Significantly different between cohorts (Mann-Whitney *U*-test, *P*=0.029).

[b] ASCh = acetylthiocholine.

The scientific value of captive-rearing juveniles for conservation initiatives may be optimized by incorporating toxicologic evaluations in prerelease health assessments. Establishing baselines for captive-reared populations is necessary to interpret future sampling results and allows captive-reared animals to be used as sentinels of ecosystem health.

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