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Authors: Laura Adamovicz, John Griffioen, Anthony Cerreta, Gregory A. Lewbart, and Matthew C. Allender

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TISSUE ENZYME ACTIVITIES IN FREE-LIVING EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*)

Laura Adamovicz, D.V.M., John Griffioen, D.V.M., Anthony Cerreta, B.S., Gregory A. Lewbart, M.S., V.M.D., Dipl. A.C.Z.M., Dipl. E.C.Z.M. (ZHM), and Matthew C. Allender, D.V.M., M.S., Ph.D., Dipl. A.C.Z.M.

Abstract: Plasma biochemical enzymes are commonly assayed as part of a diagnostic evaluation for zoological species, but their interpretation is complicated by a lack of knowledge about tissue of origin in many reptiles. This study evaluated tissue specificity of six biochemical enzymes (alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP], creatine kinase [CK], gamma-glutamyl transferase [GGT], and glutamate dehydrogenase [GLDH]) in 10 tissues (skeletal muscle, cardiac muscle, lung, liver, gallbladder, pancreas, gastrointestinal tract, kidney, spleen, and reproductive tract) from 10 free-living eastern box turtles (*Terrapene carolina carolina*). CK activity was highest in skeletal muscle, cardiac muscle, and gastrointestinal tract; GLDH and ALT activities were highest in liver, kidney, and gallbladder; ALP and GGT activities were elevated in kidney and gastrointestinal tract; and AST was relatively nonspecific, with significantly higher activity in the cardiac muscle, liver, kidney, skeletal muscle, and gallbladder compared to other tissues ($P < 0.05$). These results serve as a first step toward improving clinical interpretation of plasma biochemistry panels in box turtles.

Key words: biochemistry enzyme activity, eastern box turtle *Terrapene carolina carolina*, reptile, tissue specificity.

INTRODUCTION

The biochemistry panel is an important diagnostic test for assessing organ integrity and function in veterinary species.[12] Evaluation of serum or plasma enzyme activity is one component of the biochemistry panel used to identify targets for further diagnostic testing or therapeutic intervention. Serum or plasma enzyme activity depends largely on the amount of enzyme present within different cells, the intracellular location of the enzyme, the degree of cellular leakage, the rate of excretion from the body, and the type, duration, and severity of tissue insult.[12] Elevation of enzyme activities in plasma or serum can indicate increased cellular leakage or enzymatic induction secondary to disease processes, while decreased activity can be consistent with diminished functional tissue mass.[13,37]

Clinical interpretation of biochemistry panels and subsequent case management depends largely on knowledge about tissues of origin for each enzyme, which can be highly species specific. The tissue specificity of serum and/or plasma biochemical enzymes has been experimentally determined in a number of mammals and birds as well as a few fish and a single amphibian species.[5,8,10–12,15,16,22,25,31,32,37,38,45,46,48,50] However, the sources of plasma biochemistry enzymes have been determined in only five reptile species to date, including loggerhead sea turtles (*Caretta caretta*), Kemp's Ridley sea turtles (*Lepidochelys kempii*), green iguanas (*Iguana iguana*), yellow rat snakes (*Pantherophis* [*Elaphe*] *obsoleta quadrivitatta*), and American alligators (*Alligator mississippiensis*).[6,9,42,40,47] With over 10,000 species in the class Reptilia, there is a clear need for additional research to improve diagnostic interpretation and clinical management of reptiles.

Eastern box turtles (*Terrapene carolina carolina*) are common within the pet trade and in zoological collections, while free-living populations are experiencing range-wide declines. Primary causes of these declines include anthropogenic factors, such as habitat destruction/fragmentation, vehicle collisions, overharvesting for the pet trade, and subsidization of mesopredators.[21] Diseases, such as ranavirus, have also been recognized for their potential to impact population stability.[2–4,19,23,29,33,44] Recent veterinary research efforts have focused on characterizing health and disease epidemiology of wild and zoo-maintained individuals in order to clarify the role of disease in eastern box turtle conservation.[7,14,28,30,36,44] As a result, hematology, plasma biochemistry, protein electrophoresis, and hemoglobin-binding protein values have been reported for this species.[1,24,34,43] However, tissues of origin for plasma biochemistry enzymes have not been determined for box turtles, complicating

From the Wildlife Epidemiology Laboratory, University of Illinois, 2001 South Lincoln Avenue, Urbana, IL 61802, USA (Adamovicz and Allender); and the College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27606, USA (Griffioen, Cerreta, and Lewbart). Correspondence should be directed to Dr. Adamovicz, (laadamovicz@gmail.com).

the clinical interpretation and diagnostic utility of these values.

The objective of this study was to determine tissues of origin for six common biochemical enzymes in free-living eastern box turtles. The hypotheses were that 1) activity of creatine kinase and aspartate aminotransferase will be highest in skeletal and cardiac muscle, 2) activity of glutamate dehydrogenase will be highest in the liver, and 3) activity of alanine aminotransferase and alkaline phosphatase will be detected in multiple tissues and will be relatively non–tissue specific.

MATERIALS AND METHODS

Study Design

A sample size calculation (http://www.openepi.com) determined that eight individuals were necessary to detect a 100 IU/g difference in enzyme activity between organs (standard deviation = 50 IU/g, power [β] = 0.8, α = 0.05). This difference was targeted based on previous research.[9] A total of 10 animals were included in the study to maintain adequate sample size while allowing for some data loss due to machine error during chemistry analysis.

To reduce the use of live animals in research, this study collected tissues from free-living eastern box turtles that were euthanized due to irreparable skull fractures associated with vehicular trauma. Turtles were selected for inclusion in the study if immediate euthanasia was deemed necessary due to grave prognosis; if injuries were confined to the skull, forelimbs, and marginal scutes with no accompanying coelomic breaches; and if they appeared otherwise healthy on physical examination and gross necropsy. Exclusion criteria included small size (<250 g), administration of medications prior to euthanasia, and a >24-hr interval between euthanasia and tissue collection.

Sample Collection

All procedures involving live animals were approved by the North Carolina State University College of Veterinary Medicine (NCSU-CVM) Institutional Animal Care and Use Committee (protocol #16-049-O). Free-living eastern box turtles were opportunistically presented to the NCSU-CVM Turtle Rescue Team at various time intervals following vehicle collisions. Complete physical examinations were performed by two individuals (JG and AC). Turtles meeting the selection criteria of the study were euthanized using pentobarbital (Beuthanasia-D Special 390 mg IV, Merck & Co., Inc., Madison, NJ 07940, USA) injected intravenously via the subcarapacial sinus, brachial vein, or jugular vein. Euthanized turtles were placed on a heating pad until a heartbeat was no longer detectable using Doppler ultrasonography (Parks Medical Electronics Inc., Aloha, OR 97078, USA). Turtles were then shipped overnight on ice packs to the University of Illinois College of Veterinary Medicine (UI-CVM).

At UI-CVM, a full gross necropsy was performed on each turtle, and samples of the following organs were collected: skeletal muscle (pectoralis), cardiac muscle (combined atria and ventricle), lung, liver, gallbladder (emptied of bile), pancreas, gastrointestinal tract (equal portions of stomach, small intestine, and colon), spleen, kidney, and reproductive tract (equal portions of ovary and oviduct for females and of testis for males). Connective tissue and blood vessels were removed, and tissue samples were trimmed to 0.5 g if possible. Tissues were rinsed with sterile water and frozen at –20°C for 1–3 mo.

Sample Processing

Thawed tissues were immersed in 4°C sterile water at a ratio of 10 ml/g (5 ml per 0.5 g tissue) and homogenized using a Polytron homogenizer (Type P10/35 with power control unit [PCU-2-110], Brinkmann Instruments, Westbury, NY 11590, USA) with a sterilized saw-toothed rotor/stator at 6,000 rpm for 20 sec on ice. Homogenized samples were further disrupted using a sonicator (Model W-225R, Heat Systems–Ultrasonic Inc., Haverhill, MA 01835, USA) with a microtip probe at 45% amplitude for 30 sec on ice. Disrupted samples were centrifuged at 2,000 *g* for 15 min at 4°C. Supernatants were poured off and stored at 4°C. Pellets were resuspended in 10 ml/g 4°C sterile water, and the homogenization, sonication, and centrifugation steps were repeated. The second supernatant was added to the first, and resuspension, homogenization, sonication, and centrifugation were repeated a third time. The final volume of all three combined supernatants was recorded.

Chemistries were performed within 3 hr of sample processing using a commercial chemistry analyzer (AU680 Chemistry System, Beckman Coulter, Brea, CA 92821, USA). Enzyme activities (IU/L) were determined for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), gamma glutamyl transferase (GGT), and glutamate dehydrogenase (GLDH). These activities were multiplied by the total

volume of supernatant (in L) and divided by the original weight of the tissue (in g) to determine enzyme activity per gram of tissue (IU/g).

Statistical Analyses

Distribution for each biochemical analyte was evaluated for normality using histograms, q-q plots, skewness, kurtosis, and the Shapiro-Wilk test. Summary statistics (median, range, 25th–75th percentiles) were determined for each analyte and grouped by tissue. The Friedman rank sum test was applied to determine whether enzyme activity was significantly different between tissues. Post hoc testing using the Nemenyi multiple comparison test from the "PMCMR" package was utilized to evaluate pairwise differences.[41] The Nemenyi post hoc test adjusts critical values based on the number of comparisons being performed, thus controlling for family-wise error and eliminating the need for *P*-value adjustments.[39] The activities of CK and AST in each tissue were compared between turtles with limb/shell fractures and those without using a series of Mann-Whitney *U*-tests with a post hoc Bonferroni correction to control for family-wise error. All statistical analyses were conducted in R version 3.4.3 at an alpha level of 0.05.

RESULTS

Six adult male turtles and four adult females were included in this study. Admission dates to the NCSU-CVM Turtle Rescue Team ranged from 14 May 2016 to 09 August 2017. All animals presented with significant head trauma, including skull fractures, mandibular fractures, and bilateral proptosis, and prognosis for recovery and release was considered grave. Four turtles presented with concurrent forelimb fractures or minor carapacial fractures, but no coelomic breaches or damage to internal organs were detected. Euthanasia was performed for humane purposes in each case. Gross necropsy abnormalities were not identified, though one female was gravid.

The chemistry analyzer failed to provide results in some cases, potentially due to the presence of interfering substances within the tissue homogenates. Results were not provided for eight GLDH tests (one kidney, one liver, one gallbladder, one GI tract, two pancreas, and two female reproductive tract samples), two ALP tests (female reproductive tracts), one ALT test (liver), and four CK tests (one kidney sample and three pancreas samples).

All data were nonnormally distributed (Shapiro-Wilk, $P < 0.05$), and each Friedman test was significant ($P < 0.05$), indicating statistically significant differences in enzyme activity between tissues. Post hoc testing was conducted to test differences between each tissue combination.

CK activity was detectable in every tissue and was significantly higher in skeletal muscle compared to liver ($P < 0.0001$), gallbladder ($P = 0.03$), pancreas ($P = 0.02$), spleen ($P < 0.0001$), and reproductive tract ($P = 0.002$) (Fig. 1, Table 1). It was significantly higher in cardiac muscle compared to liver ($P < 0.0001$), spleen ($P < 0.0001$), and reproductive tract ($P = 0.03$) and higher in the GI tract compared to liver ($P = 0.0005$) and spleen ($P = 0.0005$). CK activity was most concentrated in the skeletal muscle, with a median value 1,961 IU/g higher than that of the cardiac muscle. The median value in the cardiac muscle was 569 IU/g higher than that of the GI tract, which in turn was 377–463 IU/g higher than all other tissues. CK activity was not significantly different between turtles with forelimb/shell fractures and those without in any tissue ($P > 0.05$).

AST activity was detectable in every tissue and was significantly higher in cardiac muscle compared to lung ($P = 0.0005$), pancreas ($P = 0.02$), spleen ($P < 0.0001$), and reproductive tract ($P = 0.0005$). It was higher in liver and kidney compared to lung ($P = 0.01$, $P = 0.01$), spleen ($P = 0.0005$, $P = 0.0007$), and reproductive tract ($P = 0.01$, $P = 0.01$) and higher in the skeletal muscle and gallbladder compared to the spleen ($P = 0.004$, $P = 0.02$). The difference in median enzyme activity between tissues ranged from 8.9 to 31.7 IU/g, indicating that AST has similar activity profiles in many tissues. AST activity was not significantly different between turtles with forelimb/shell fractures and those without in any tissue ($P > 0.05$).

ALT activity was detectable at low levels in every tissue and was significantly higher in liver compared to lung ($P < 0.0001$), pancreas ($P = 0.0004$), spleen ($P = 0.0006$), reproductive tract ($P = 0.0003$), and gastrointestinal tract ($P = 0.02$). It was higher in kidney and gallbladder compared to lung ($P < 0.0001$, $P < 0.0001$), pancreas ($P = 0.02$, $P = 0.02$), spleen ($P = 0.03$, $P = 0.02$), and reproductive tract ($P = 0.02$, $P = 0.017$). The maximum difference in median enzyme activity between tissues was 6.3 IU/g, indicating that ALT is not highly concentrated in any one tissue.

GLDH activity was detectable in every tissue and was higher in liver compared to skeletal muscle ($P < 0.0001$), cardiac muscle ($P =$

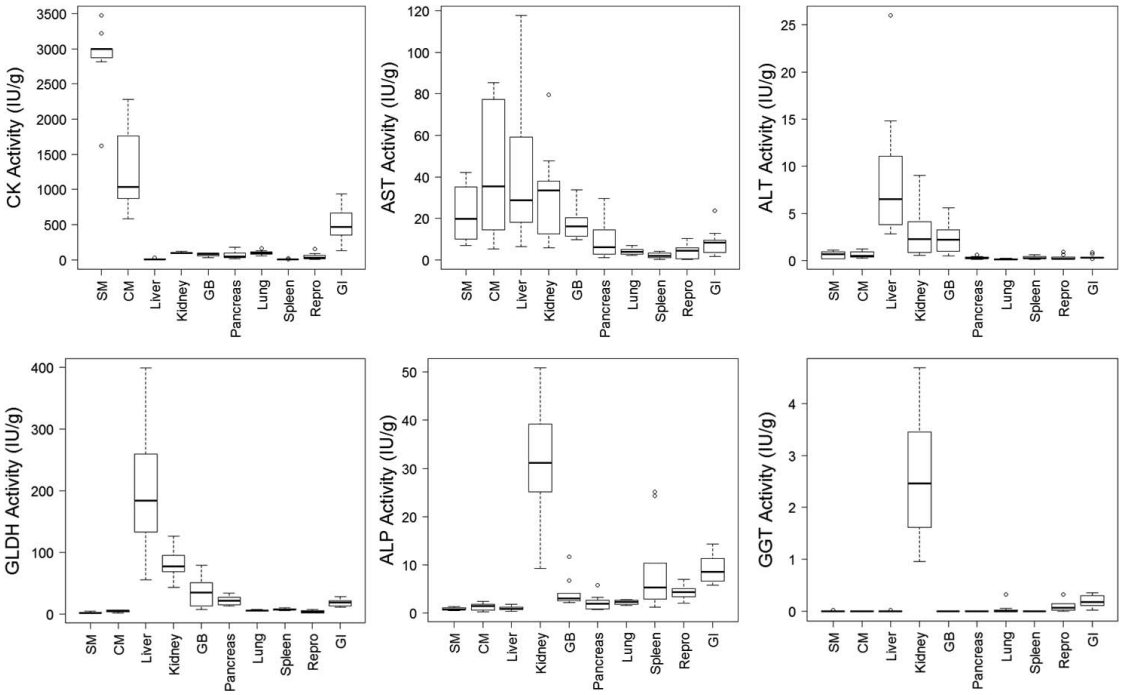

Figure 1. Enzyme activity in 10 eastern box turtle (*Terrapene carolina carolina*) tissues. CK indicates creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLDH, glutamate dehydrogenase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; SM, skeletal muscle; CM, cardiac muscle; GB, gallbladder; Repro, reproductive organs; GI, gastrointestinal tract.

0.0008), lung ($P = 0.008$), and reproductive tract ($P = 0.002$). Activity was higher in the kidney compared to skeletal muscle ($P = 0.0005$), cardiac muscle ($P = 0.01$), and reproductive tract ($P = 0.02$). Finally, activity was higher in the gallbladder and pancreas compared to the skeletal muscle ($P = 0.008$, $P = 0.02$). GLDH activity was most concentrated in the liver, with a median value 106 IU/g higher than the kidney. The median activity in the kidney was 42 IU/g higher than the gallbladder, and the gallbladder median was within 33 IU/g of the remaining tissues.

ALP activity was detectable in all tissues and was higher in kidney compared to skeletal muscle ($P < 0.0001$), cardiac muscle ($P < 0.0001$), lung ($P = 0.02$), liver ($P < 0.0001$), and pancreas ($P = 0.01$). Activity was also higher in the GI tract compared to skeletal muscle ($P = 0.0001$), cardiac muscle ($P = 0.001$), and liver ($P = 0.001$) and higher in the reproductive tract than the skeletal muscle ($P = 0.03$). ALP activity was most concentrated in the kidney, with a median value 22 IU/g higher than that of the GI tract. The median value of the GI tract was within 7.7 IU/g of all other tissues.

GGT activity was detected in at least one sample from the skeletal muscle, liver, kidney, lung, GI tract, and reproductive tract. Consistent detection in >90% of samples was achieved only for the kidney, GI tract, and reproductive tract. GGT activity was not detected in cardiac muscle, gallbladder, pancreas, and spleen. Statistical analyses were restricted to tissues where GGT activity was present. Activity was higher in the kidney than the skeletal muscle ($P = 0.0008$), lung ($P = 0.01$), and liver ($P = 0.0008$) and higher in the GI tract than the skeletal muscle ($P = 0.04$) and liver ($P = 0.04$). GGT activity was detected primarily in the kidney, with a median value 2.28 IU/g greater than that of the GI tract and 2.38 IU/g greater than the reproductive tract, respectively.

DISCUSSION

This study documents tissues of origin for six common plasma biochemical enzymes in eastern box turtles. Results indicate that CK activity is greatest in skeletal muscle, cardiac muscle, and GI tissue; ALT and GLDH activities are highest in the liver, kidney, and gallbladder; ALP and GGT activities are elevated in the kidney and GI tract; and AST activity is higher in cardiac muscle, liver, kidney, skeletal muscle, and gallbladder compared to other tissues. Within box turtle

tissues, enzyme activity was high (>1,000 IU/g) for CK, moderate (100–1,000 IU/g) for GLDH, lower (10–100 IU/g) for AST and ALP, and lowest (<10 IU/g) for GGT and ALT. No enzymes were fully specific to a single tissue. Differences in methodology, enzymes considered, and tissues included prohibit direct comparison of enzyme activities between studies, but when overall patterns of activity are compared between species, a few clear patterns emerge.

CK activity is consistently present at high levels in skeletal and cardiac muscle in every species evaluated and is also detectable in the smooth muscle from organs such as the GI tract.[5,6,9–11,16,22,25,31,32,37,38,40,42,45,47] Tissue distributions for GGT and GLDH are also highly conserved across taxa, with greatest activity levels in the kidney and liver, respectively.[5,6,9,10–12,16,22,25,31,32,37,38,40,42] Eastern box turtle tissue specificity is consistent with other vertebrates for these three enzymes.

AST activity is concentrated in hepatic and muscle tissue in most animals, though the specific site with the highest activity is species dependent and low to moderate activity is commonly present in all tissues.[5,10–12,15,16,22,25,31,32,37,38,45,50] In box turtles and other reptiles, renal AST activity can be comparable to that of the liver, cardiac muscle, and skeletal muscle.[6,9,40,42,47] Low overall tissue specificity for AST appears somewhat universal, though it still maintains clinical utility in many species.[9,45]

ALT activity tends to divide mammals into one of two groups: those where the highest activity is present in the liver (canids, felids, primates, etc.) and those where the highest activity is present in skeletal and cardiac muscle (ungulates).[12,16,22,32] In reptiles, ALT activity either is significantly higher in the kidney than any other tissue (American alligators and yellow rat snakes) or, like most avian species, has a low overall activity that is concentrated in the liver and kidney (sea turtles, eastern box turtles, and green iguanas).[6,9,37,38,40,42,47] The diagnostic sensitivity and specificity for ALT is typically greatest for mammals with high hepatic concentrations of this enzyme, though its clinical utility for box turtles and other reptiles cannot be immediately ruled out without further studies.[12,13,16,32]

ALP activity is present at low to moderate levels in the tissues of mammals and birds, and it is typically most concentrated in the kidney and GI tract.[12,16,32,37,38,49] Eastern box turtles and yellow rat snakes follow this pattern, but most other ectothermic vertebrates have a somewhat different tissue distribution.[42] In green iguanas, ALP activity is concentrated in the kidney, while in American alligators, it is highest in the intestine, followed by the cardiac muscle, liver, and kidney.[9,47] ALP activity is highest in the spleen of loggerheads and the lung of Kemp's Ridley sea turtles.[6,40] Theoretically, ALP activity is also elevated in the bone of all species due to the presence of a bone isozyme; however, few studies have confirmed this due to the difficulty associated with bone homogenization protocols.[6,12,13]

Eastern box turtle tissue enzyme distribution is generally similar to other species. While these findings provide an important first step toward understanding plasma biochemical analysis in box turtles, tissue enzyme distribution alone does not fully explain patterns of enzyme elevation in plasma.[12,13,38] The location of the enzyme within the cell; rate of clearance from the plasma; nature of the enzyme (leakage vs induction); type, duration, and severity of cellular insult; relative mass of affected tissue; and characteristics of the source tissue can also affect plasma enzyme activity and contribute significantly to the clinical value of a particular enzyme.[12,13]

Diagnostically relevant enzymes are typically found in the cytosol (CK, ALT, AST), mitochondria (GLDH, AST), and/or cellular membranes (GGT, ALP) within their tissues of origin.[12,13] This intracellular organization dramatically influences the degree of leakage in response to cellular insult. Cytosolic enzymes can elevate in the plasma due to mild forms of cell injury, such as reversible increases in membrane permeability associated with inflammation. In contrast, enzymes anchored to cellular components, such as GLDH, are typically not released from cells unless necrosis and subsequent cellular lysis are present.[12,13] The type and degree of cellular damage can therefore significantly impact the pattern of plasma enzyme activities and affect the diagnostic sensitivity of certain enzymes. In box turtles, cytosolic enzymes, including CK, ALT, and AST, may be expected to rise in plasma in response to injury in their tissues of origin. Mitochondrial enzymes, such as GLDH, may rise with hepatocyte necrosis, though changes in liver-associated cytosolic enzymes (ALT, AST) may be more sensitive for liver damage.

Diagnostic sensitivity is also highly dependent on the half-life of an enzyme in serum or plasma. Enzymes that are rapidly eliminated from the body have limited clinical utility because of their narrow window of elevation.[12,13,38] There is currently no information on plasma half-lives of

Table 1. Summary enzyme activity data in 10 tissues from eastern box turtles (*Terrapene carolina carolina*).[a]

	Median	Quartiles	Range
CK (IU/g)			
SM	3000[A,D]	2,892.45, 3,000	1,620.9–3,470.4
CM	1038.68[A,C,D]	869.03, 1,611.41	584.25–2,277.6
Liver	5.73[B]	4.41, 9.87	2.07–27.12
Kidney	99.6	84.84, 107.46	84.27–123.69
GB	79.71[B,C,D]	53.49, 93.26	30.42–96.12
Pancreas	47.28[B,C,D]	32.43, 81.68	9.96–180.24
Lung	92.03	76.39, 108.61	55.38–163.38
Spleen	7.7[B]	7.22, 8.5	3.39–22.65
Repro	25.82[B,D]	12.76, 58.78	7.59–154.56
GI	469.2[C,D]	363.11, 638.55	129.36–938.4
GLDH (IU/g)			
SM	1.80[B]	1.38, 2.69	1.17–4.38
CM	4.97[B,D]	3.92, 5.47	1.47–6.29
Liver	183.96[A]	133.17, 259.8	55.66–398.91
Kidney	77.25[A,C]	68.28, 94.71	42.89–126.06
GB	34.95[A,D]	13.11, 50.6	7.16–78.89
Pancreas	21.25[A,D]	15.78, 25.05	13.02–33.17
Lung	5.38[B,C,D]	4.46, 5.84	3.94–6.71
Spleen	7.32	6.83, 8.21	5.28–9.77
Repro	3.28[B,D]	1.95, 5	1.63–7.35
GI	18.12	12.79, 21.66	10.58–27.58
AST (IU/g)			
SM	19.77[A,D]	10.4, 32.28	6.96–42.15
CM	35.415[A]	15.39, 71.12	5.25–85.41
Liver	28.68[A,C]	18.5, 54.26	6.45–117.66
Kidney	33.585[A,C,D]	16.65, 37.94	5.73–79.5
GB	16.17[A]	11.85, 20.45	9.9–33.78
Pancreas	6.15[B,C,D]	2.95, 12.25	1.05–29.55
Lung	3.78[B,D]	2.75, 4.96	2.31–7.02
Spleen	1.845[B]	1.14, 3.31	0.33–4.26
Repro	4.35[B,D]	1.25, 5.69	0.18–10.41
GI	8.4	3.8, 9.32	1.68–23.85
ALP (IU/g)			
SM	0.78[B]	0.78, 1.01	0.45–1.35
CM	1.425[B,D]	0.83, 1.79	0.21–2.46
Liver	0.81[B,D]	0.74, 1.2	0.36–1.77
Kidney	31.14[A]	26.22, 37.46	9.27–50.82
GB	2.985	2.55, 3.92	2.13–11.7
Pancreas	1.935[B,C,D]	1.13, 2.54	0.72–5.73
Lung	2.325[B,C,D]	1.9, 2.64	1.59–2.73
Spleen	5.28	3.12, 9.43	1.2–25.11
Repro	4.305[A,D]	3.58, 5.01	2.07–6.96
GI	8.505[A,C]	6.7, 11.13	5.82–14.37
ALT (IU/g)			
SM	0.66	0.26, 0.92	0.15–1.11
CM	0.5	0.34, 0.86	0.21–1.2
Liver	6.48[A]	3.81, 11.04	2.79–25.98
Kidney	2.27[A,C]	0.85, 3.91	0.54–9
GB	2.21[A,C]	1.12, 3.08	0.51–5.58
Pancreas	0.24[B]	0.22, 0.34	0.09–0.63
Lung	0.14[B]	0.09, 0.15	0.09–0.24
Spleen	0.24[B]	0.18, 0.39	0.09–0.63
Repro	0.18[B]	0.15, 0.3	0.09–0.93
GI	0.3[B,C]	0.27, 0.3	0.18–0.87

Table 1. Continued.

	Median	Quartiles	Range
GGT (IU/g)			
SM	0[B]	0, 0	0–0.03
CM	0	0, 0	0–0
Liver	0[B]	0, 0	0–0.03
Kidney	2.46[A]	1.67, 3.44	0.96–4.68
GB	0	0, 0	0–0
Pancreas	0	0, 0	0–0
Lung	0[B,C]	0, 0.02	0–0.33
Spleen	0	0, 0	0–0
Repro	0.075	0.038, 0.15	0–0.33
GI	0.18[A,C]	0.13, 0.28	0.03–0.36

[a] Median enzyme activities with different superscript letters are significantly different between tissues ($P < 0.05$). CK indicates creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLDH, glutamate dehydrogenase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; SM, skeletal muscle; CM, cardiac muscle; GB, gallbladder; Repro, reproductive organs; GI, gastrointestinal tract.

diagnostic enzymes in reptiles, which is a significant limitation for clinical interpretation of biochemistry panels in this taxon.

Biologic behavior and function of biochemistry enzymes is another important consideration for determining clinical relevance. Most enzymes evaluated in the present study (CK, AST, ALT, GLDH) are leakage enzymes with relatively straightforward associations between plasma activity and tissue activity. However, the activities of enzymes such as GGT and ALP can be significantly up-regulated during disease states due to enzyme induction despite fairly low levels in normal tissues.[12,13,38] For example, GGT activity is typically highest in the kidney, but plasma levels of GGT elevate most reliably in response to biliary disease in mammals.[12] It is therefore much more difficult to infer potential clinical utility for these enzymes from their relative tissue specificity. Future studies pairing histologic diagnoses with plasma biochemistry changes will be necessary to assess the diagnostic capabilities of induction enzymes in reptiles.

Finally, tissue characteristics are important to consider when interpreting the results of tissue enzyme specificity studies. Enzymes produced by the excretory organs (kidney and GI tract) tend to be expelled in waste instead of circulated in plasma.[12,13] For example, GGT activity is highly concentrated in the kidney in most species, but plasma GGT levels do not elevate in response to renal disease. Instead, GGT activity rises in the urine.[12,13] Urinary GGT can be used as a diagnostic test to evaluate renal health in mammals, but interpretation of urinary GGT in reptiles and birds may be complicated by the semisolid consistency of the urine, mixing of urine and feces within the cloaca, and the presence of postrenal urine modification within the urinary bladder or colon.[27] Similar to the excretory organs, the activity of enzymes within the brain can be masked by the blood-brain barrier despite this organ having high activity for CK in multiple species.[6,12,13]

In box turtles, activities of GGT, ALT, ALP, AST, and GLDH are high in the kidney, while CK and ALP are high in the GI tract; however, damage to the kidney or GI tract may result in loss of enzymes into the feces and urine instead of elevation in the plasma. ALT and GLDH may therefore be primarily markers for liver disease/damage, though the overall concentration of ALT in box turtle tissues is low; AST may be a good marker for muscle and liver insult, and CK may be best associated with muscle damage. GGT and ALP are inducible enzymes, and it is difficult to determine their diagnostic utility without further testing.

Future studies may advance reptile clinical pathology and improve the interpretation of plasma biochemistry panels. Next steps include determining tissue enzyme specificity in more species, quantifying enzyme clearance rates from the plasma, assessing association between clinical pathology changes and histologic evidence of disease in retrospective case-control studies, experimentally inducing organ damage and monitoring plasma enzyme activity changes, and potentially using proteomic approaches to identify novel enzyme targets in order to improve detection and diagnosis of organ disease in reptiles.

Limitations of the present study are related largely to methodology. This study utilized tissues from free-living eastern box turtles with irreparable skull damage due to vehicular trauma in order to avoid euthanasia of otherwise healthy animals. Comparison of muscle damage enzyme activity (CK and AST) between turtles with concurrent limb/shell fractures and those with skull fractures alone demonstrated no difference between groups, supporting the validity of this approach. Pentobarbital was utilized for euthanasia of the turtles in this study, and while alteration of tissue enzyme activities secondary to administration of this drug is unexpected, the possibility cannot be fully ruled out. Several other tissue specificity studies have also used tissues collected through wildlife rehabilitation centers, and while there are some drawbacks associated with potential drug administration and incompletely characterized health status, this appears to be a good way to obtain scientifically useful data while reducing animal use in science.[6,22,35,40] While all turtles included in the study appeared outwardly healthy, the presence of infectious disease cannot be fully ruled out. Box turtles exercise behavioral fever in response to pathogen challenge, and open areas such as roads are popular basking sites.[20] It is possible that at least some of the animals included in this study were struck on roads while basking due to illness instead of crossing the road for other behavioral reasons, such as foraging or nesting.[26] The results of this study may have been slightly different if completely healthy, uninjured animals were utilized, though such a study design would have been hard to ethically justify.

This study may have been improved by considering paired enzyme activities in antemortem plasma samples and tissues to determine relative concentrations in each compartment. This was not pursued because the presence of traumatic injuries in the study animals would have likely altered the plasma distribution of muscle leakage enzymes and decreased the generalizability of results to healthy individuals.

Additional limitations include the short delay between death and tissue collection, which was necessary due to shipment of animal remains. All turtles were maintained on ice after death, and it is unclear if or how this <24-hr delay may have impacted tissue enzyme activity. Similarly, tissue samples were frozen at –20°C for 1–3 mo until sample processing and chemistry analysis. Previous research in humans and rats indicates that the plasma biochemical enzymes evaluated in this study remain stable in serum at –20°C for up to 3 mo.[17,18] Freezing samples for variable periods of time, sometimes even longer than 3 mo, is commonly performed in tissue specificity studies, and results obtained in the present study are comparable to those of previous research efforts.[5,6,8,22,25,40,45,47,49] Finally, though the targeted sample size for each organ in this study was eight, only six male and four female reproductive tracts were available for testing. Due to small sample size, effects of sex on enzyme activity in other organs could not be rigorously assessed.

This study determined the activity levels of six common biochemical enzymes in 10 eastern box turtle tissues. The findings are generally comparable to other species, with some potentially important exceptions. This research lays the groundwork for future studies that will improve clinical interpretation of reptile biochemistry panels and ultimately advance reptilian veterinary practice.

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