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A Pilot Study Investigating Plasma Protein Electrophoresis in One Anuran and Six Urodelan Species

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ABSTRACT: As threats to amphibian health increase, there is a growing need for diagnostic tools to assess and monitor their health status. Plasma protein electrophoresis has proven to be useful in other nonmammalian species. It enables quantification of protein fractions in plasma that may be altered in various disease processes, and is therefore useful in narrowing down differential diagnoses and detecting inflammation, in combination with other modalities such as biochemical and hematologic testing. The amphibian electrophoretogram must be defined before baseline reference intervals are obtained across species. Agarose gel electrophoresis was performed on plasma samples collected from presumed clinically normal individuals of one anuran and six urodelans: *Osteopilus septentrionalis* ($n=2$), *Gyrinophilus porphyriticus* ($n=1$), *Notophthalmus viridescens* ($n=1$), *Eurycea guttolineata* ($n=2$), *Amphiuma tridactylum* ($n=2$), *Cryptobranchus alleganiensis* ($n=5$), and *Siren lacertina* ($n=6$). The electrophoretograms varied in number of fractions between each species; however, the number of fractions was consistent within a species. An albumin migrating fraction was consistently observed in all species. A prealbumin migrating fraction was identified in species that primarily use organs other than skin for respiration. This study provides preliminary examples of a normal plasma protein electrophoretogram for seven amphibian species. Further studies quantifying reference intervals and identification of protein fractions will help establish protein electrophoresis as a useful tool in amphibian health investigations.

Key words: Agarose gel electrophoresis, albumin, amphibian, globulin, salamander.

With at least 41% of amphibian species threatened with extinction, and increasing rates of population decline (IUCN 2020), it is critical to develop diagnostic tools to monitor amphibian health. Plasma protein electrophoresis may be used to assess health status through quantitating protein fractions in the blood that may be altered during the acute phase response and various disease processes. It is a well-described technique in mammalian and avian species and is becoming more commonly used in reptiles (Cray 2021). However, investigation in amphibian species is lacking.

Plasma protein electrophoresis separates proteins in the blood based on their physical properties including electrical charge. In semiautomated agarose gel electrophoresis, plasma proteins separate into fractions including albumin and three types of globulins (alpha, beta, and gamma; Cray 2021). Albumin is the predominant fraction in normal plasma samples. Some nonmammalian species also have an additional fraction anodic to the albumin fractions, conventionally referred to as a prealbumin fraction (Cray 2021). Changes in the levels of the protein fractions may occur in the acute phase response and in many disease processes such as inflammation, neoplasia, and dehydration.

This tool may be particularly useful in amphibians for two reasons. First, it can be performed on very small amounts of blood (2 μm). Because of their frequently small size, blood volumes that can safely be obtained from amphibians are often less than 0.5 mL (Forzán et al. 2017). For example, in an average-sized Cuban tree frog (*Osteopilus septentrionalis*) with a body weight of 57 g, approximately 0.24 mL of blood can be taken from a clinically ill animal and 0.48 mL can be collected from a clinically healthy animal (Forzán et al. 2017). Second, protein electrophoresis provides a much more accurate measurement of albumin than do commonly used methods such as bromocresol green (Cray 2021). The goal of our study was to provide example plasma protein electrophoretograms from presumed clinically

normal individuals of seven diverse amphibian species including one frog (Anura) and six salamanders (Urodela).

Species examined included adult *O. septentrionalis* (n=2), *Siren lacertina* (greater siren, n=6), *Gyrinophilus porphyriticus* (spring salamander, n=1), *Notophthalmus viridescens viridescens* (eastern newt, n=1), *Eurycea guttolineata* (three-lined salamander, n=2), *Amphiuma tridactylum* (three-toed amphiuma, n=2), and *Cryptobranchus alleganiensis alleganiensis* (hellbender, n=5). The hellbenders were sampled in the wild from two separate sites in Tennessee, US (Middle and East) under Tennessee Wildlife Resources Agency (TWRA) Permit 1691. All other individuals were held in a human-managed setting. Cuban tree frogs and greater sirens were collected by Florida Fish and Wildlife Conservation Commission, the spring salamander was collected from the North Carolina Wildlife Resources Commission, the eastern newt was collected from Tennessee under TWRA permit 1504, and three-toed amphiumas were collected from Louisiana under Louisiana Department of Wildlife and Fisheries permit WDP-20-077. Three-lined salamanders were captive bred and purchased from Indoor Ecosystems, LLC (Whitehouse, Ohio, USA).

Animals (except hellbenders sampled in the wild) were transported to and housed in the Johnson Animal Research and Teaching Unit at the University of Tennessee, Knoxville (UTK), Tennessee, US, under UTK-IACUC protocol 2723 (greater sirens) or UTK-IACUC protocol 2395 (all others). Greater sirens and three-toed amphiumas were individually housed in tanks at room temperature (approximately 20 C). All other species were individually housed in separate containers within an environmental growth chamber (Conviron, Winnipeg, Manitoba, Canada).

The spring salamander, three-lined salamanders, and three-toed amphiumas were skin swabbed (10 passes along the ventrum and five along the bottom of each foot) for *Batrachochytrium dendrobatidis* (*Bd*) by qPCR upon entry into the laboratory and confirmed to be negative using techniques similar to those described by Boyle et al. (2004). Cuban tree frogs, eastern newts, and sirens were heat treated for *Bd* infection upon arrival into the laboratory, using 10 d in an environmental growth chamber at 30 C (Chatfield and Richards-Zawacki 2011; Bletz 2013). Animals were then skin swabbed and confirmed to be negative for *Bd* via the same qPCR.

Following heat treatment, individuals were acclimated over 2 wk to a temperature of 14 C. Environmental chambers were also set to a light-dark cycle that corresponded with the season associated with this temperature (spring and fall = 14 h dark) and kept at a humidity of >90%. Water changes were performed once every 3 d using dechlorinated and aged tap water for individuals housed in environmental growth chambers. Health checks consisting of brief visual examination were performed twice daily. Animals that had no external lesions, had appropriate body condition, and were displaying behavior normal for the species were considered healthy. All human-managed individuals were fed 4% of their body mass 24 h before each water change. The eastern newt and greater sirens were fed bloodworms (*Chironomus plumosus*); three-toed amphiumas were fed earthworms (*Eisenia fetida*) and bloodworms; Cuban tree frogs and spring salamander were fed crickets (*Gryllodes sigillatus*), and the three-lined salamanders were fed fruit flies (*Drosophila melanogaster*). All species were euthanized within 90 d postentry into the laboratory, except for the sirens, which were in human-managed care for approximately 20 mo before euthanasia. Table 1 lists the experimental duration for each of the human-managed species before blood collection.

Blood was collected from the ventral tail vein of hellbenders using a heparinized syringe. A skin swab was collected at the time of blood collection and processed for *Bd* qPCR following Hardman (2020). All animals were confirmed to be qPCR negative for *Bd*. A heparinized syringe was used to collect blood from the tail veins of sirens that were anesthetized with benzocaine hydrochloride dissolved in dechlorinated water. The remaining individuals were euthanized using transdermal exposure to 70% benzocaine (Leary et al. 2020), and blood samples were collected immediately postmortem, by cardiocentesis using a heparinized capillary tube. Postmortem sampling was used because these

TABLE 1. Experimental duration of each human-managed species of amphibians before blood collection for plasma protein electrophoresis and the month each sample was collected. The species were *Osteopilus septentrionalis* (Cuban tree frog), *Siren lacertina* (greater siren), *Gyrinophilus porphyriticus* (spring salamander), *Notophthalmus viridescens* (eastern newt), *Eurycea guttolineata* (three-lined salamander), *Amphiuma tridactylum* (three-toed amphiuma), and *Cryptobranchus alleganiensis* (hellbender).

Species	Experimental duration	Sampling month
Osteopilus septentrionalis	75 d	April
Siren lacertina	18 mo	February
Gyrinophilus porphyriticus	59 d	July
Notophthalmus viridescens	44 d	February
Eurycea guttolineata	59 d	October
Amphiuma tridactylum	59 d	December
Cryptobranchus alleganiensis (TN1, TN3, TN4)[b]	NA[a]	June
Cryptobranchus alleganiensis (TN2)	NA	August
Cryptobranchus alleganiensis (TN5)	NA	May

[a] NA = not applicable.
[b] TN1, TN2, TN3, TN4, and TN5 = animal IDs.

were control animals from a larger study investigating chytrid infection, and manipulation for blood drawing may have altered the study results because of stress induction. Additionally, in the smaller species (spring salamander, eastern newt, and three-lined salamander), blood collection was not possible antemortem because of their small size.

Collection of blood antemortem versus immediately postmortem was not expected to have an effect on results, as previous reports have shown it takes hours for the acute phase proteins to be altered following a stimulus (Cray et al. 2009). There is a lack of information on the effect of benzocaine exposure on plasma protein levels in veterinary species. Studies have shown other types of anesthetics to have an effect on biochemical values (Pierozan et al. 2017; Khokhlova et al. 2022). Therefore, it is possible that the euthanasia method may have affected testing results.

Blood samples from all species were kept on ice initially. Within 2 h, blood was centrifuged in a microcapillary tube at 2,170 × G for at least 3 min to separate plasma. Samples of 2 µL plasma from each animal were analyzed in accordance with instructions provided by the Helena QuickGel system with the use of Split Beta gels (Helena Laboratories, Inc., Beaumont, Texas, USA). Samples were run in duplicate. A known human control sample (Helena Laboratories, Inc.) and a known abnormal sample from a dog with multiple myeloma were run as controls to ensure consistency between gels. The percentages of each fraction were determined by the use of gel densitometry and QuickGel Software provided by Helena.

To compare the variation in fraction sizes between species to the variation in fraction sizes within a species, the compositional mean of the presumed albumin fraction as well as the fraction immediately cathodic (towards the positive electrode) to albumin was calculated for the species with samples from more than one individual (hellbenders, Cuban tree frogs, greater sirens, three-lined salamanders, and three-toed amphiumas). These two fractions were chosen because they were consistently present among all species. For species with only one individual tested (*Gyrinophilus porphyriticus* and *Notophthalmus viridescens*), the observed compositional data were used as the compositional mean. The metric variance, an analog of sample variance used with compositional data (van den Boogart and Tolosana-Delgado 2013), was determined among all species mean compositions and compared with the metric variance for the compositions of hellbenders (n=5) and sirens (n=6). All analyses

were performed using the 'Real compositions' package in R (version 4.1.1; van den Boogart and Tolosana-Delgado 2013).

Plasma protein fractions from each species are presented in Table 2. Representative plasma electrophoretograms from each amphibian species along with the controls are presented in Figure 1. Plasma protein profiles varied greatly in the number of protein fractions among amphibian species, with nine fractions observed in the Cuban tree frog (Fig. 1A) and three in the greater siren (Fig. 1D).

All amphibian samples examined had a presumed albumin migrating fraction, which represented the largest fraction in all species aside from the hellbender. Four species, Cuban tree frogs (Fig. 1A), three-toed amphiumas (Fig. 1B), eastern newt (Fig. 1C), and greater sirens (Fig. 1D), had a fraction migrating anodic to the presumed albumin fraction. The number and size of fractions cathodic to the presumed albumin fraction varied among species. The metric variance of the presumed albumin fraction and the fraction cathodic to albumin among all species was 0.44, among hellbenders it was 0.13, and among sirens it was 0.08. This confirmed, as expected, that among-species variation in plasma protein profiles was larger than within-species variation.

Our study provides preliminary electrophoretograms from presumed clinically normal individuals of seven amphibian species. This work demonstrates 1) the feasibility of using plasma protein electrophoresis across a diversity of amphibian species, and 2) the potential of plasma protein electrophoresis to provide species-specific health profiles. It also provides the groundwork for future studies to develop reference intervals for these and other amphibian species so that plasma protein electrophoresis can become a useful diagnostic tool for health assessment in amphibians.

We identified marked variation in plasma protein electrophoretograms between amphibian species, even within the same order. Similarly, interspecific variations have been described in elasmobranch and avian species (Roman et al. 2013; Morón-Elorza et al. 2022). Fraction number and size varied among each of the species examined, and fraction size varied within members of the same species. Previous studies have shown similar intraspecific variation (Cray 2021). Overall, variation in fraction sizes between species was determined to be greater than variation in fraction sizes within a species. This highlights that species-specific reference intervals need to be developed before using electrophoretograms as a diagnostic tool for a particular amphibian species to have. Interestingly, each of the four species in which we found a prealbumin migrating fraction (Cuban tree frog, three-toed amphiuma, eastern newt, and greater siren) primarily rely on organs other than their skin (external gills, lungs, or a combination of both) for respiration (Wells 2007). The species that lacked this fraction (hellbender, three-lined salamander, and spring salamander) primarily rely on their skin for respiration, with varying but lesser degrees of buccopharyngeal membrane respiration (Wells 2007). The significance of this finding is uncertain. A prealbumin fraction has been reported in healthy individuals of some bird and reptile species (Zaias and Cray 2002), and prealbumin has been associated with binding thyroxine and retinol in avian species (Lumeji 2008). Therefore, this result might suggest a difference in one or both of these parameters among the two groups, and perhaps is linked to respiration; this is presently only speculative. Important next steps to elucidate the biological significance of the prealbumin migrating fraction will be to assess whether this pattern holds across larger sample sizes and to use mass spectrometry to confirm identification of proteins within each fraction.

Protein electrophoretograms of all species appeared to have what would be, based on the migration characteristics of well-documented species (Cray 2021), an albumin fraction. Previous studies using various methods of electrophoresis, as well as staining reaction techniques, have also suggested that amphibian species have one–two albumin migrating peaks, which can vary in size (Frieden et al. 1957; Herner and Frieden 1960; Chen 1967, 1970; Francis et al. 1985; Chiesa et al. 2006; Young et al. 2012, 2014; Cray 2021). This is an important finding, because changes in albumin levels can

TABLE 2. Plasma protein fraction results for clinically healthy individual amphibians from seven species, determined by plasma protein electrophoresis.

Species	Animal ID	Figure 1 (Y/N)[a]	Percentage of total protein								
			Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9
Osteopilus septentrionalis	CTF1	Y	4.7	28.9	17.9	18.8	11.6	6.7	4.2	3.2	5.1
Osteopilus septentrionalis	CTF2	N	1.5	34.7	15.9	19.0	11.9	4.9	4.3	1.9	5.7
Siren lacertina	SL1	N	22.1	45.8	31.9	NA[b]	NA	NA	NA	NA	NA
Siren lacertina	SL3	Y	19.1	59.2	21.6	NA	NA	NA	NA	NA	NA
Siren lacertina	SL4	N	29.1	44.9	25.9	NA	NA	NA	NA	NA	NA
Siren lacertina	SL5	N	23.4	50.9	25.6	NA	NA	NA	NA	NA	NA
Siren lacertina	SL6	N	19.1	58.8	22.1	NA	NA	NA	NA	NA	NA
Siren lacertina	SL8	N	30.8	41.9	27.3	NA	NA	NA	NA	NA	NA
Gyrinophilus porphyriticus	GY1	Y	32.6	30.8	8.6	13.5	8.6	5.8	NA	NA	NA
Notophthalmus viridescens	D2	Y	19.9	32.9	13.2	28.3	5.6	NA	NA	NA	NA
Eurycea guttolineata	EG4	N	64.7	12.1	19.2	3.9	NA	NA	NA	NA	NA
Eurycea guttolineata	EG5	Y	61.0	12.7	22.4	3.8	NA	NA	NA	NA	NA
Amphiuma tridactylum	AT1	Y	4.3	34.5	6.1	19.5	14.8	20.8	NA	NA	NA
Amphiuma tridactylum	AT2	N	5.2	31.6	8.4	19.4	19.2	16.0	NA	NA	NA
Cryptobranchus alleganiensis	TN1	Y	31.1	15.9	14.3	38.7	NA	NA	NA	NA	NA
Cryptobranchus alleganiensis	TN2	N	35.7	10.3	19.9	34.0	NA	NA	NA	NA	NA
Cryptobranchus alleganiensis	TN3	N	22.5	17.1	24.7	35.7	NA	NA	NA	NA	NA
Cryptobranchus alleganiensis	TN4	N	19.9	16.2	20.3	43.6	NA	NA	NA	NA	NA
Cryptobranchus alleganiensis	TN5	N	36.5	13.6	20.5	29.4	NA	NA	NA	NA	NA

[a] Y = yes; N = no.
[b] NA = not applicable.

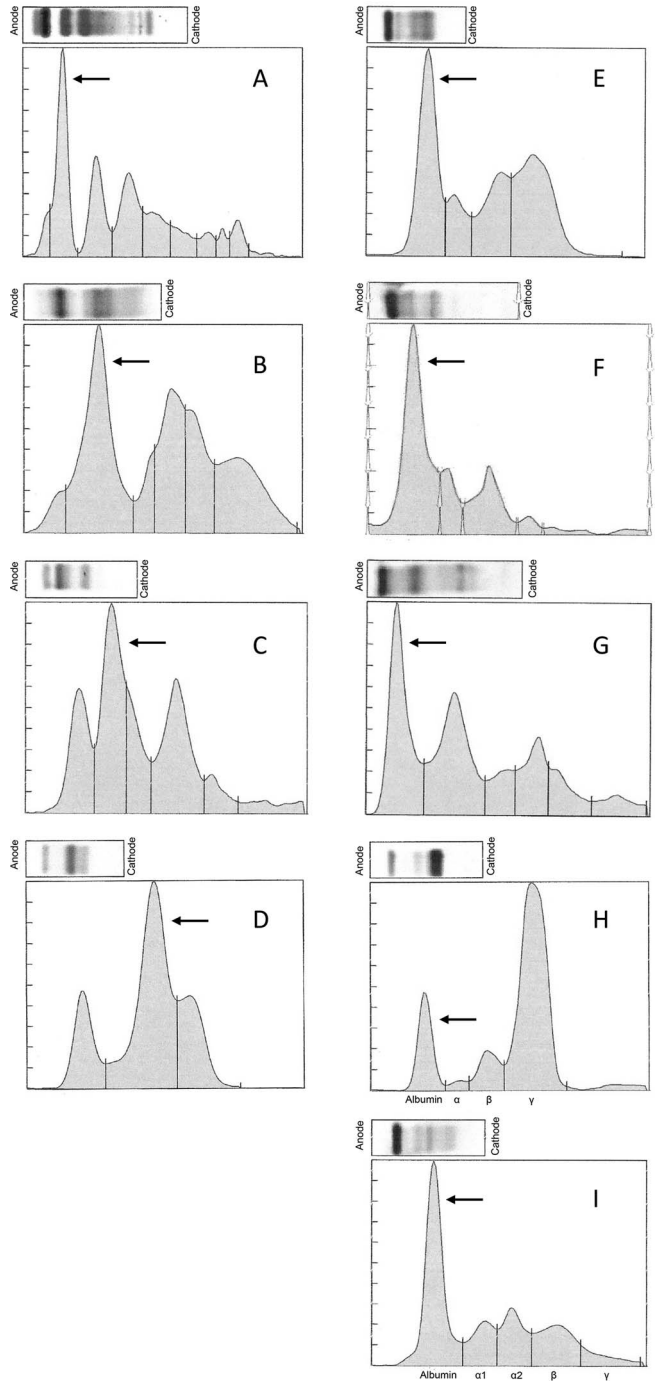

FIGURE 1. Representative agarose gel electrophoresis (AGE) electrophoretograms of heparinized plasma from (A) a Cuban tree frog (*Osteopilus septentrionalis*), (B) a three-toed amphiuma (*Amphiuma tridactylum*), (C) an eastern newt (*Notophthalmus viridescens*), (D) a greater siren (*Siren lacertina*), (E) a hellbender (*Cryptobranchus alleganiensis*), (F) a three-lined salamander (*Eurycea guttolineata*), (G) a spring salamander (*Gyrinophilus porphyriticus*), plus (H) serum from a dog with multiple myeloma, and (I) serum from a human control. Arrows represent the presumed albumin migrating fraction.

be useful in diagnosing many pathologic processes, including active inflammation; dehydration; and liver, gastrointestinal, or renal disease; as well as malnutrition (Forzán et al. 2017).

A limitation of this technique in smaller species was that it could only be performed postmortem. Limitations of our study include small sample size and lack of supporting biochemical and hematologic analysis to evaluate health status. Future inclusion of multiple animals of different sexes, development stages, and geographic locations over multiple seasons will be needed to enable development of reference intervals. Additionally, classification of the protein fractions by mass spectrometry is necessary. Future studies using these techniques will help us to understand the significance and utility of plasma protein electrophoresis in amphibians.

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