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Source: Journal of Zoo and Wildlife Medicine, 45(2):298-305.
Published By: American Association of Zoo Veterinarians
DOI: http://dx.doi.org/10.1638/2013-0147R1.1
URL: http://www.bioone.org/doi/full/10.1638/2013-0147R1.1

Journal of Zoo and Wildlife Medicine 45(2): 298–305, 2014

EFFECTS OF RANAVIRUS INFECTION OF RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*) ON PLASMA PROTEINS

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Abstract: Ranavirus is an emerging disease that infects fish, amphibians, and reptiles. Ranavirus induces an inflammatory response leading to death in many susceptible species. Red-eared sliders (RES; *Trachemys scripta elegans*) are vulnerable to ranavirus infection and are economically significant chelonians kept in the pet trade and utilized in research. Early identification of RES with inflammatory diseases would allow for isolation of affected individuals and subsequent disease investigation, including molecular testing for ranavirus. Validation of an inexpensive, clinically relevant, and reproducible diagnostic test that detects inflammation in turtles is needed. Although commonly used, plasma protein electrophoresis to detect an inflammatory acute-phase protein response has not been evaluated in a controlled environment in turtles with experimentally induced inflammatory disease. The objective of this study was to measure plasma protein fractions by electrophoresis to determine if an acute-phase protein response occurs in RES during infection with a frog virus 3–like ranavirus (FV3-like virus) isolated from a chelonian. A Bradford assay and agarose gel electrophoresis (AGE) were performed using plasma collected during a study of the effect of temperature on the pathogenesis of ranavirus in RES. In RES at the time of viremia, total albumin ($ALB_{mg/ml}$) and albumin to globulin ratio were significantly lower and β-globulin percentage was significantly higher in RES exposed to ranavirus ($n = 4$) as compared to matched, uninfected RES ($n = 8$). In the last sample collected prior to death, total protein ($TP_{mg/ml}$), $ALB_{mg/ml}$, α-globulin percentage, and total α-globulin ($\alpha_{mg/ml}$) were significantly lower in RES exposed to ranavirus ($n = 4$) than control individuals ($n = 8$). In summary, FV3-like virus induces a decrease in plasma albumin concentration at the onset of viremia and decreases in $TP_{mg/ml}$, $ALB_{mg/ml}$, and $\alpha_{mg/ml}$ concentrations prior to death in RES as measured by AGE.

Key words: Acute-phase protein, albumin, electrophoresis, globulin, ranavirus, turtle.

INTRODUCTION

The acute-phase response is a biological reaction to trauma or infection that helps to control injury induced by invading pathogens, mediate tissue damage, and promote a rapid return to homeostasis.[2] During this response, hepatic and extrahepatic synthesis of a heterogeneous group of acute-phase proteins is altered. Acute-phase protein concentrations can be a crucial part of a diagnostic evaluation in species where more classic signs of inflammation are not present.[2,7,17–21,23,24,41]

The diagnosis of inflammation typically is not problematic in equine, canine, and feline species because an inflammatory leukogram often can be detected by performing a complete blood count (CBC). Acute-phase protein concentrations are used in these species to confirm that inflammation is occurring, guide treatment, and provide useful prognostic information.[23] In cattle, however, the peripheral blood leukocyte response to inflammation is blunted, often not showing significant changes until severe disease is present. As a result, evaluation of acute-phase proteins to detect inflammatory disease in bovine medicine is commonplace.[18] Peripheral blood cell counts are routinely performed in reptiles; however, leukocytosis is not reliably observed during markedly inflammatory disease processes.[39] In the turtles used in this study, there were no significant differences between CBC results of ranavirus-infected turtles as compared to uninfected turtles despite the histologic evidence of marked inflammation (splenitis, vasculitis, and pneumonia) in the infected turtles.[1]

The inability to detect inflammation using CBC results has led to utilization of acute-phase proteins for routine monitoring of inflammatory disease in several reptilian species.[5,14,35,37,43] However, scientific data to support this practice are limited. One controlled study evaluated hepatic transcription of acute-phase proteins in Chinese soft-shelled turtles (*Trionyx sinensis*) experimentally exposed to the freshwater bacteria *Aeromonas hydrophila*.[43] These animals had hepatic transcript changes consistent with an acute-phase response during inflammation, including an increase in fibrinogen transcripts and a decrease in albumin transcripts.[43] Hepatic biopsy to measure

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acute-phase protein transcripts using real-time quantitative polymerase chain reaction (PCR) is invasive and likely would be inappropriate as a diagnostic tool in conservation and patient management efforts. Instead, there has been a call for a well-controlled investigation into the validity of using plasma protein electrophoresis for detecting an inflammatory acute-phase protein response in reptiles.[15,39]

In mammals, protein electrophoresis is commonly performed using serum, rather than plasma. It is known that the correlation between canine plasma and serum protein electrophoresis is poor.[8] Most studies using electrophoresis evaluate plasma proteins in reptiles, as plasma is easier to collect because of patient size and limited sample volume. Therefore, development of plasma protein reference intervals in reptiles, distinct from serum protein reference intervals, is needed.

Comparison of different plasma electrophoretic methods has shown that, for healthy red-eared sliders (RES; *Trachemys scripta elegans*) and green iguana (*Iguana iguana*), agarose gel electrophoresis (AGE) is preferred.[15] Therefore, this study uses AGE to compare plasma acute-phase protein concentrations in RES ($n = 4$) at several time points during a controlled experimental infection with a frog virus 3–like ranavirus (FV3-like virus) that was isolated from a chelonian. The results from infected RES were compared to those from matched uninfected RES ($n = 8$). Additional clinical and histologic data from this experiment have been published and are available for review.[1]

FV3-like virus induces a marked fibrinoid necrosis, heterophilic splenitis, vasculitis, and mild to moderate heterophilic pneumonia in experimentally infected RES.[1] Because the histologic lesions in FV3-like virus–infected RES are inflammatory, it is reasonable to propose that ranavirus would induce an acute-phase protein response. Ranavirus is a clinically important member of the Iridoviridae family that has been associated with significant morbidity and mortality in wild amphibian and chelonian populations.[16,28] It has recently been placed on the World Organization for Animal Health reportable disease list for amphibians, and increased work in understanding its pathogenesis and epidemiology have been undertaken.[22,26,27,42] Early detection of inflammation would help ongoing conservation efforts by prompting researchers to isolate and test affected animals for ranavirus infection.

The specific biological hypothesis evaluated in this study is that ranavirus will induce an acute-phase protein response in RES, which can be measured by AGE as a decreased albumin percentage and concentration and increased globulin fraction percentages and concentrations.

MATERIALS AND METHODS

All animal activities were approved by the University of Illinois Animal Use and Care Committee (Protocol 11050). Heparinized plasma from 12 captive-raised RES was collected during an investigation into the effect of temperature on the pathogenesis of ranavirus after intramuscular exposure to 5×10^6 of a 50% tissue culture infective dose ($TCID_{50}$) FV3-like virus, as described elsewhere.[1] Control turtles were similarly inoculated with the same volume of sterile saline. In brief, 12 adult female captive-raised RES were acclimated to 22°C for 1 wk. Four individuals were included in the exposed group with two individuals in the control group matched to each exposed individual (a total of eight control individuals). Heparinized plasma was collected from all individuals 7 and 4 days prior to FV3-like virus or saline inoculation. Following exposure, all individuals were phlebotomized twice weekly until the members of the exposed group were euthanized because of severity of clinical signs (nasal and ocular discharge, oral plaques, lethargy, leg swelling, and dermal ulcerations). Two matched individuals from the control group were euthanized concurrently with each exposed individual. Samples were stored at –20°C for 1 yr prior to testing.

Because the initial experimental exposure of the turtles was completed before conception of this study, alteration in sample size was not possible without complete recapitulation of the previous study. Although the sample size was small, utilizing the plasma from the previously completed study was deemed likely sufficiently sized to detect changes in the key proteins of interest and provide data that can be used to inform future experimental efforts.

Heparinized plasma from a quarter horse foal (*Equus ferus caballus*) with septic arthritis and pooled heparinized plasma collected during a surveillance effort of apparently healthy wild eastern ornate box turtles (*Terrapene carolina carolina*) also were used for procedural control purposes. These control plasma samples were stored at –80°C for 2 mo (horse) or –20°C up to 1 yr (box turtle) prior to testing.

AGE was used as described elsewhere.[25] Briefly, the plasma was thawed and recentrifuged to produce platelet-poor plasma, which was diluted

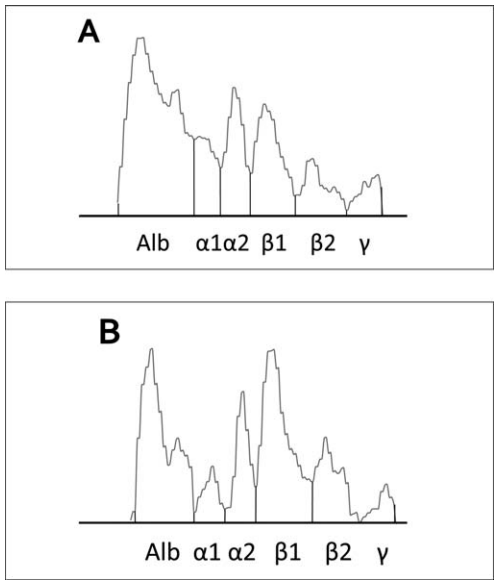

Figure 1. Two representative electrophoretograms, both from a single red-eared slider (turtle 4). Plasma protein fractions were separated using agarose gel electrophoresis. The densitometry tracings are from a plasma sample collected during the pre-exposure time period (**A**) and at the onset of frog virus 3–like viremia as documented by quantitative polymerase chain reaction analysis of peripheral blood (**B**). Alb indicates albumin; α, α-globulin; β, β-globulin; γ, γ-globulin

1 : 20 in PBS. A Bradford assay (to determine total plasma protein concentration) and plasma electrophoresis were performed in duplicate. Resulting electrophoresis gels were stained with Coomassie blue and the relative densities of separated protein fractions were determined using a Kodak 440CF (Eastman Kodak Co., Rochester, New York 14650, USA) and Carestream Molecular Imaging Software (Carestream Health, Rochester, New York 14608, USA) with manual assignment of protein fractions similar to previously reported work.[15] These data were imported into Excel software (Microsoft Corporation, Redmond, Washington 98052, USA) and protein fraction concentrations in the plasma samples were calculated.

Four time periods were defined for statistical evaluation: 1) pre-exposure (Pre-exp): both samples collected prior to the date of exposure were measured and averaged; 2) postexposure (Post-exp): a single plasma collection 11 days after the date of exposure, before clinical signs of disease in the exposed group; 3) Viremia: the first sample with quantitative PCR–verified viremia in the exposed turtles and the same time in the matched control turtles (7–21 days prior to death); 4) Last: the last sample collected before death (1–4 days prior to death). Insufficient plasma was available from one control turtle to test at Post-exp.

Evaluation of Pre-exp data for descriptive purposes utilized a Shapiro-Wilks test for normality followed by calculation of mean and composition of a histogram as recommended by the American Society for Veterinary Clinical Pathology for $10 < n < 20$.[12] Given that the exposed group had an $n = 4$, comparison of exposed to nonexposed groups was accomplished using a one-tailed Mann-Whitney U test to detect differences at a level of P-value < 0.05. As all tests could not be run at the same time, an aliquot of equine and an aliquot of pooled box turtle heparinized plasma were included as a procedural control with each run, and an interrun reliability coefficient was calculated as recommended by Nunnally.[34] Fleiss's recommendations that a reliability coefficient >0.75 is excellent, 0.40–0.70 is fair to good, and <0.40 is less than desirable were used.[9] Statistical evaluation was performed using GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, California 92037, USA), GraphPad StatMate 2 (GraphPad Software, Inc.), and Excel.

RESULTS

Figure 1 illustrates representative electrophoretograms of plasma collected from a turtle prior to ranavirus infection (Fig. 1A) and at the time that ranavirus was detected by quantitative PCR (Fig. 1B). For statistical analysis, protein fractions were classified as albumin, α-globulin (α), β-globulin (β), and γ-globulin (γ). Subclassifications of α and β fractions were not analyzed because adequate differentiation between α1- and α2-globulin or β1- and β2-globulin fractions was lacking in several of the samples. Interrun reliability coefficients were excellent (>0.90) for all measured values except for total albumin ($ALB_{mg/ml}$) and $\gamma_{mg/ml}$, which were fair to good (0.59 and 0.48, respectively).

Total protein ($TP_{mg/ml}$) concentrations are presented in Figure 2A. Note that $TP_{mg/ml}$ was significantly lower in exposed turtles as compared to control turtles in the last sample collected prior to death (Last, $P = 0.014$).

The percentages of protein fractions separated using AGE in exposed and control RES are available in Supplemental Figure S1. There was a significantly larger decrease in $ALB_{\%}$ from Pre-exp to Viremia values in exposed turtles as compared to control turtles ($P = 0.008$; Fig. S1A). In exposed turtles, $\alpha_{\%}$ was significantly lower at Last ($P = 0.014$; Fig. S1B) and $\beta_{\%}$ was

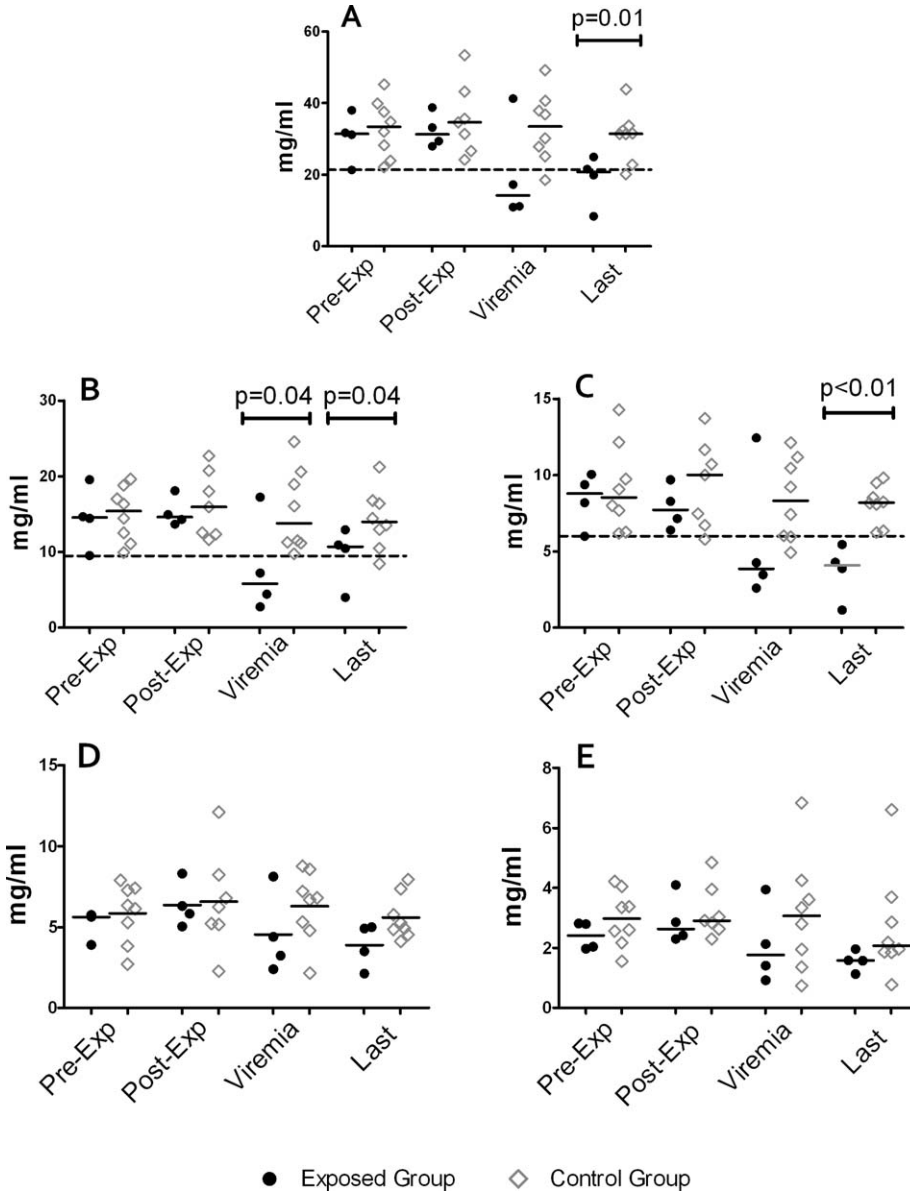

Figure 2. Plasma protein concentrations at clinically relevant time points in frog virus 3–like ranavirus–exposed and control red-eared sliders acclimated to 22°C. Total protein (**A**) concentrations were determined using a Bradford assay. Albumin (**B**), α-globulin (**C**), β-globulin (**D**), and γ-globulin (**E**) concentrations were calculated from total protein and agarose gel electrophoresis data. The black solid lines indicate the mean. The black dashed line indicates lower limit of observed pre-experimental (Pre-exp) values where clinically relevant. The solid bracket indicates comparison of control and exposed turtles. Post-exp indicates postexposure.

significantly higher at Viremia ($P = 0.024$; Fig S1C) as compared to controls. No significant difference between ranavirus-exposed and control turtles was detected in $\gamma_{\%}$ (Fig. S1D).

Calculated protein fractions from electrophoretic analysis of the plasma samples and $TP_{mg/ml}$ measurements are presented in Figure 2. $ALB_{mg/ml}$ was significantly lower at Viremia and Last in exposed turtles as compared to control turtles ($P =$ 0.036 and 0.036, respectively; Fig. 2B). There was a significant decrease in total α ($\alpha_{mg/ml}$) at Last in exposed turtles as compared to control turtles ($P =$ 0.002; Fig. 2C). Significant differences in $\beta_{mg/ml}$ or $\gamma_{mg/ml}$ between groups were not observed (Fig. 2D,

Table 1. A comparison of the percentage of frog virus 3–like ranavirus–exposed or control red-eared sliders with Viremia and Last plasma protein measurements outside of limits of pre-exposure ranges. Only values with significant differences between ranavirus-infected and control groups are shown.

Sample	Plasma protein fraction	Limit of pre-exposure result	% of ranavirus-exposed turtles ($n = 4$)	% of control turtles ($n = 8$)
Viremia	Albumin (mg/ml)	<9.5	75	0
	β-globulins (%)	>20.9	75	25
	Albumin/globulins	<0.71	75	25
Last	Total protein (mg/ml)	<21.4	50	12.5
	Albumin (mg/ml)	<9.5	25	12.5
	α-globulins (%)	<21.9	100	12.5
	α-globulins (mg/ml)	<6.0	100	0

E, respectively). The albumin to globulin (A : G) ratio was significantly lower at Viremia in exposed turtles as compared to control turtles ($P = 0.049$; data not shown).

No significant differences in protein concentrations were noted between the exposed and control groups at Pre-exp or Post-exp. Pre-exp data from all turtles were combined to illustrate protein concentration intervals in healthy RES maintained in a controlled, 22°C environment (Fig. S2). Because of the low number of individuals in this study, a rigorous evaluation of sensitivity and specificity of plasma protein subsets is inappropriate. However, an evaluation of which individuals were outside the Pre-exp ranges reveals some interesting findings. The relevant limits are indicated on Figures 2 and S1 as dashed horizontal lines for the protein fractions, with significant differences between FV3-like virus–infected and control turtles.

At two time points, Viremia and Last, a larger percentage of infected than uninfected RES had specific plasma protein values outside of the Pre-exp range (Table 1). At Viremia, $ALB_{mg/ml}$ lower than 9.5 mg/ml was observed in three of four exposed turtles and zero of eight control turtles (Fig. 2B); $\beta_{\%}$ values above 20.9% were observed in three of four exposed turtles and two of eight control turtles (Fig. S1C); and three of four samples from exposed turtles had an A : G ratio <0.71, whereas two of eight samples from control turtles were below this value. In samples taken just prior to death (Last), $TP_{mg/ml}$ in two of four exposed turtles and one of eight control turtles was lower than the Pre-exp range of 21.4 mg/ml (Fig. 2A); $\alpha_{\%}$ below 21.9% was observed in four of four samples from the exposed group and one of eight samples from control turtles (Fig. S1B); and all Last samples below the $\alpha_{mg/ml}$ Pre-exp range of 6.0 mg/ml were from exposed turtles (Fig. 2C).

DISCUSSION

The $TP_{mg/ml}$ as measured by a dye binding (Bradford) assay in this study correlates with the previously published total solid concentration as measured by refractometry ($r = 0.70$).[1] Both measurements observed a significant decrease in plasma protein concentrations of FV3-like virus–infected RES prior to death. Additional changes were detected in this study 1–4 days prior to death using plasma AGE: namely, there were significant decreases in $ALB_{mg/ml}$, $\alpha_{\%}$, and $\alpha_{mg/ml}$. These changes are consistent with loss of protein associated with the vasculitis noted histologically in these individuals, and suggest that one of the major disease processes of FV3-like virus infection involves third spacing of body fluid and circulatory collapse in RES.[1] However, free coelomic fluid was not observed at necropsy, and a significant increase in $\beta_{\%}$ was observed at an earlier time point (Viremia), indicating that vasculitis was not the only mechanism causing decreased $ALB_{mg/ml}$ in these turtles. In light of changes found in acute-phase protein transcripts in Chinese soft-shelled turtles infected with *A. hydrophila*, lack of protein production by hepatocytes is likely a contributing factor to the changes in plasma protein concentrations observed in this study.[43]

A classic mammalian acute-phase protein response would include a decrease in albumin and an increase in α, β, and/or γ fraction concentrations. Observational studies in chelonians suggest that decreases in all protein electrophoresis fractions occur during an acute-phase protein response in turtles.[5,35] In this study, there was a significant decrease in $ALB_{mg/ml}$ and increase in $\beta_{\%}$, but only a mild decrease in $TP_{mg/ml}$ at Viremia. A significant decrease in $TP_{mg/ml}$, $ALB_{mg/ml}$, $\alpha_{\%}$, and $\alpha_{mg/ml}$ was observed just prior to death. These changes suggest that an acute-phase protein response is occurring in ranavirus-infected RES.

No significant changes were observed in the γ values during the course of this study. The γ fraction in reptiles is primarily (although not exclusively) comprised of immunoglobulins. The lack of change in the γ fractions of ranavirus-infected turtles is not unexpected given the classically slow seroconversion process of reptiles and the naïve status of the experimental individuals.[6,29,44] Reevaluation of this particular facet of the acute-phase protein response would be appropriate in RES that had recovered from ranavirus infection or in RES afflicted with a more indolent disease process.

The evaluation of plasma protein fractions by the number of individuals outside the Pre-exp range reveals interesting findings that suggest a clinically relevant use of these variables as a diagnostic tool. It must be remembered when considering these data that they are derived from a small number of individuals and a rigorous evaluation of sensitivity and specificity is inappropriate. Additionally, it is well accepted that all analytical processes will have a degree of inherent error and that any diagnostic tool should be evaluated for observed error.[40] Measurement of plasma protein fractions in additional healthy and ranavirus-infected RES is needed to determine the predictive value of these findings in a clinical setting and produce appropriate recommendations for total allowable error.

Electrophoretic patterns of plasma proteins from a variety of turtle species have previously been published revealing species-specific patterns in plasma electrophoretograms.[4,11,33,37] This has led to the practice of speciating turtles based on their electrophoretic and protein profiles. It is interesting to note that several of the published species-specific profiles are more consistent with the ill RES than the healthy control group in this study. One excellent example can be seen in eight specimens of the European pond turtle, *Emys orbicularis*, presented by Musquera et al.[33] It is possible that individual animals used in some of the previous studies were not healthy; however, it is more likely that knowledge of the species-specific normal electrophoretic and protein profiles is required before using plasma electrophoresis to screen for an acute-phase protein response in reptiles.[41]

Comparing the observed mean values from the Pre-exp group to reference intervals published for RES, it is noted that the $TP_{mg/ml}$ and $\alpha_{mg/ml}$ were similar to published concentrations.[10,15] However, appreciable differences in $ALB_{mg/ml}$, $\beta_{mg/ml}$, and $\gamma_{mg/ml}$ were observed.[15] Several factors may explain these differences. First, the appearance of protein bands in agarose gels varies with agarose percentage, voltage, and length of electrophoresis. Second, after plasma is electrophoresed, the protein bands are stained and manually designated as ALB, α, β, and γ fractions. Differences in staining techniques and manual band assignments may have led to differences between studies. Third, seasonal differences, independent of temperature, affect innate and adaptive immunity in wild RES.[44] It is possible that seasonality affected values in the captive RES used in this study. Finally, environmental temperature affects ectotherm protein production and chelonian immunoglobulin production.[29,44] As part of this current study, turtles were intentionally housed at a suboptimal environmental temperature, which may have resulted in differences between studies.

The results of this study should be viewed as representative of response in suboptimal, yet commonly encountered, environmental conditions, as turtles in this study were maintained in an environment that prevented them from attaining their preferred internal temperature range of 24–30°C for healthy adult RES.[3,13,30,36] Multiple studies have shown that, at suboptimal temperatures, ectotherms have an altered metabolism and immune response. Marked differences in phagocytic ability, complement activity, immunoglobulin levels, and delay in seroconversion are observed at different temperatures in multiple species.[6,29,31,32,38] The acute-phase protein response should be investigated at normal and potentially elevated temperatures to better understand the plasma protein changes as a function of temperature in RES.

Significant changes were noted in plasma AGE data of FV3-like virus–infected turtles at two time points: Viremia (7–21 days prior to death) and Last (1–4 days prior to death). These changes suggest that a decrease in $ALB_{mg/ml}$ with an increase in $\beta_{\%}$ may be an early indicator of inflammatory disease in RES. Also, decreases in $TP_{mg/ml}$, $ALB_{mg/ml}$, $\alpha_{\%}$, and $\alpha_{mg/ml}$ may be observed 1–4 days prior to death due to ranavirus infection in RES. This study provides information needed to critically evaluate the plasma protein response in RES and helps to confirm the existence of an acute-phase protein response during a known inflammatory disease.

Acknowledgment: The authors would like to thank the Zuckermann laboratory at the University of Illinois College of Veterinary Medicine Pathobiology Department for providing additional laboratory equipment for this study.

LITERATURE CITED

1. Allender MC. Thesis: Characterizing the epidemiology of ranavirus in North American chelonians: diagnosis, surveillance, pathogenesis, and treatment [Internet]. 2012 [cited 2013 May 31]. Univ. of Illinois, Urbana (IL). Available from https://www.ideals.illinois.edu/handle/2142/34286

2. Bayne CJ, Gerwick L. The acute phase response and innate immunity of fish. Dev Comp Immunol. 2001;25:725–743.

3. Boyer TH, Boyer DM. Turtles, tortoises, and terrapins. In: Mader DR (ed.). Reptile medicine and surgery. 2nd ed. Saint Louis (MO): Saunders Elsevier; 2006. p. 78–99.

4. Cohen E. A comparison of the total protein and albumin content of the blood sera of some reptiles. Science. 1954;119:98–99.

5. Deem SL, Norton TM, Mitchell M, Segars A, Alleman AR, Cray C, Poppenga RH, Dodd M, Karesh WB. Comparison of blood values in foraging, nesting, and stranded loggerhead turtles (*Caretta caretta*) along the coast of Georgia, USA. J Wildl Dis. 2009;45:41–56.

6. Dessauer HC. Plasma proteins of reptilia. In: Florkin M, Sheer B (eds.). Chemical zoology, Volume IX. New York (NY): Academic Press; 1974. p. 187–216.

7. Eckersall PD, Lawson FP, Bence L, Waterston MM, Lang TL, Donachie W, Fontaine MC. Acute phase protein response in an experimental model of ovine caseous lymphadenitis. BMC Vet Res. 2007;3:35.

8. Errico G, Giordano A, Paltrinieri S. Diagnostic accuracy of electrophoretic analysis of native or defribrinated plasma using serum as a reference sample. Vet Clin Pathol. 2012;41:529–540.

9. Fleiss JL. The design and analysis of clinical experiments. 1st ed. New York (NY): John Wiley & Sons; 1986. p. 3–28.

10. Frair W. Turtle family relationships as determined by serological tests. In: Taxonomic biochemistry and serology. New York (NY): Ronald Press Co.; 1964. p. 535–544.

11. Frair W. Taxonomic relations among chelydrid and kinosternid turtles elucidated by serological tests. Copeia. 1972;1:97–108.

12. Friedrichs KR, Harr KE, Freeman KP, Szladovits B, Walton RM, Barnhart KF, Blanco-Chavez J. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet Clin Pathol. 2012;41:441–53.

13. Gatten REJ. 1974. Effect of nutritional status on the preferred body temperature of the turtles *Pseudemys scripta* and *Terrapene ornata*. Copeia. 1974:912–917.

14. Gicking JC, Foley AM, Harr KE, Raskin RE, Jacobson E. Plasma protein electrophoresis of the Atlantic loggerhead sea turtle, *Caretta caretta*. J Herpetol Med Surg. 2004;14:13–18.

15. Giménez M, Saco Y, Pato R, Busquets A, Martorell JM, Bassols A. Plasma protein electrophoresis of *Trachemys scripta* and *Iguana iguana*. Vet Clin Pathol. 2010;39:227–235.

16. Green DE, Converse KA, Schrader AK. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Ann N Y Acad Sci. 2002;969:323–339.

17. Harr KE, Harvey J, Bonde R, Murphy D, Lowe M, Menchaca M, Haubold E, Francis-Floyd R, David Murphy D. Comparison of methods used to diagnose generalized inflammatory disease in manatees (*Trichechus manatus latirostris*). J Zoo Wildl Med. 2006;37:151–159.

18. Heegaard PM, Godson DL, Toussaint MJ, Tjørnehøj K, Larsen LE, Viuff B, Rønsholt L. The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. Vet Immunol Immunopathol. 2000;77:151–159.

19. Heegaard PMH, Klausen J, Nielsen JP, González-Ramón N, Piñeiro M, Lampreave F, Alava MA. The porcine acute phase response to infection with *Actinobacillus pleuropneumoniae*. Haptoglobin, c-reactive protein, major acute phase protein and serum amyloid A protein are sensitive indicators of infection. Comp Biochem Physiol B Biochem Mol Biol. 1998; 119:365–373.

20. Hulten C, Sandgren B, Skioldebrand E, Klingeborn B, Marhaug G, Forsberg M. The acute phase protein serum amyloid a (SAA) as an inflammatory marker in equine influenza virus infection. Acta Vet Scand. 1999;40:323–333.

21. Jensen LE, Hiney MP, Shields DC, Uhlar CM, Lindsay AJ, Whitehead AS. Acute phase proteins in salmonids: evolutionary analyses and acute phase response. J Immunol. 1997;158:384–392.

22. Johnson AJ, Pessier AP, Jacobson ER. Experimental transmission and induction of ranaviral disease in western ornate box turtles (*Terrapene ornata ornata*) and red-eared sliders (*Trachemys scripta elegans*). Vet Pathol. 2007;44:285–297.

23. Kjelgaard-Hansen M, Jacobsen S. Assay validation and diagnostic applications of major acute-phase protein testing in companion animals. Clin Lab Med. 2011;31:51–70.

24. Kovacs BM, Toussaint MJ, Gruys E, Fabian IB, Szilagyi L, Janan J, Rudas P. Evaluation of goose serum amyloid A acute phase response by enzyme-linked immunosorbent assay. Acta Vet Hung. 2007;55:349–357.

25. Krizek DM, Rick ME. Agarose gel electrophoresis of proteins. Current protocols in cell biology. 2002;15:6.7.1–6.7.13.

26. Latney LV, Klaphake E. Selected emerging diseases of amphibia. Vet Clin North Am Exot Anim Pract. 2013;16:283–301.

27. Lesbarreres D, Balseiro A, Brunner J, Chinchar VG, Duffus A, Kerby J, Miller DL, Robert J, Schock

DM, Waltzek T, Gray MJ. Ranavirus: past, present and future . Biol Lett. 2011;8:481–483.

28. Mao J, Hedrick RP, Chinchar VG. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology. 1997;229:212–220.

29. Maung RT. Immunity in the tortoise *Testudo ibera*. J Pathol Bacteriol. 1963;85:51–66.

30. McArthur S, Barrows M. General care of chelonians. In: McArthur S, Wilkinson R, Meyer J (eds.). Medicine and surgery of tortoises and turtles. 1st ed. Oxford, United Kingdom: Blackwell Publishing Ltd; 2004. p. 87–107.

31. Merchant ME, Roche C, Elsey RM, Prudhomme J. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). Comp Biochem Physiol B Biochem Mol Biol. 2003;136:505–513.

32. Mondal S, Rai U. In vitro effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, *Hemidactylus flaviviridis*. Comp Biochem Physiol A Mol Integr Physiol. 2001; 129:391–398.

33. Musquera S, Massegú J, Planas J. Blood proteins in turtles (*Testudo hermanni*, *Emys orbicularis*, and *Caretta caretta*). Comp Biochem Physiol A Physiol. 1976;55:225–230.

34. Nunnally JC. Psychometric theory. 2nd ed. New York (NY): McGraw-Hill; 1978.

35. Osborne AG, Jacobson ER, Bresette MJ, Singewald DA, Scarpino RA, Bolten AB. Reference intervals and relationships between health status, carapace length, body mass, and water temperature and concentrations of plasma total protein and protein electrophoretogram fractions in Atlantic loggerhead sea turtles and green turtles. J Am Vet Med Assoc. 2010; 237:561–567.

36. Parmenter RR, Avery HW. The feeding ecology of the slider. In: Gibbons JW (ed.). Life history and ecology of the slider turtle. Washington (DC): Smithsonian Institution Press; 1990. p. 257–266.

37. Perrault JR, Miller DL, Eads E, Johnson C, Merrill A, Thompson LJ, Wyneken J. Maternal health status correlates with nest success of leatherback sea turtles (*Dermochelys coriacea*) from Florida. PloS One. 2012;7:e31841.

38. Raffel TR, Rohr JR, Kiesecker JM, Hudson PJ. Negative effects of changing temperature on amphibian immunity under field conditions. Funct Ecol. 2006; 20:819–828.

39. Sykes JM, Klaphake E. Reptile hematology. Vet Clin North Am Exot Anim Pract. 2008;11:481–500.

40. Vap LM, Harr KE, Arnold JE, Freeman KP, Getzy K, Lester S, Friedrichs KR. ASVCP quality assurance guidelines: control of preanalytical and analytical factors for hematology for mammalian and nonmammalian species, hemostasis, and crossmatching in veterinary laboratories. Vet Clin Pathol. 2012;41: 8–17.

41. Werner LL, Reavill DR. The diagnostic utility of serum protein electrophoresis. Vet Clin North Am Exot Anim Pract. 1999;2:651–662.

42. World Organisation for Animal Health. Diseases listed by OIE. Chapter 1.3, article 1.3.4. In: Aquatic Animal Health Code 2008. Paris, France: OIE; 2008. p. 2.

43. Zhou X, Wang L, Feng H, Guo Q, Dai H. Acute phase response in Chinese soft-shelled turtle (*Trionyx sinensis*) with *Aeromonas hydrophila* infection. Dev Comp Immunol. 2011;35:441–451.

44. Zimmerman LM, Paitz RT, Vogel LA, Bowden RM. Variation in the seasonal patterns of innate and adaptive immunity in the red-eared slider (*Trachemys scripta*). J Exp Biol. 2010;213:1477–1483.

Received for publication 1 July 2013