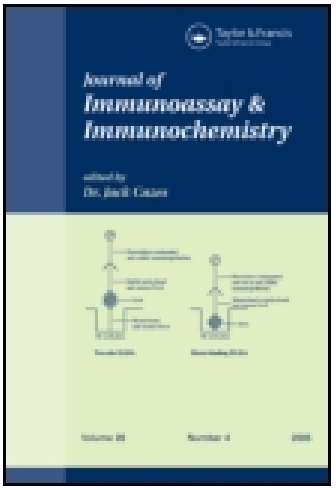

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INFLUENCE OF COLLECTION TIME ON HEMATOLOGIC AND IMMUNE MARKERS IN THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*)

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□ *Crocodilians are important keystone species and indicators of environmental health. Much remains unknown, however regarding utility of field-collected crocodilian blood samples for ecologic assessments. Field sampling sites are also often distant to analysis centers, necessitating development of new techniques and panels of assays that will yield environmentally relevant data. Stability and viability of hematological and immunological indices have been of particular interest for linking ecosystem health to biomarkers in resident species. In this study, we investigated the effect of time at analysis post-blood sampling at 4 and 24 hr on a panel of potential biomarkers in alligator blood. Our results suggest alligator blood samples can be reliably evaluated for both hematologic and immunologic profile 24 hr after sampling.*

Keywords alligator, blood collection, hematology, lymphocyte separation, proliferation, time

INTRODUCTION

The ability of organisms to fight off infectious pathogens is critical of individuals and species. Crocodilians are composed of at least 23 different species scattered throughout temperate and tropical climates worldwide, and represent an ancient group of reptiles occupying a unique evolutionary and ecological niche among vertebrates.[1,2] While much research has

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investigated various aspects of behavior[3], ecology,[4,5] and reproduction and sex determination,[6–8] anecdotal observations along with some previous research,[9,10] has suggested a robust immune system. Nevertheless, relative to other higher vertebrate species, limited information is available about the crocodilian immune system. Coinciding with their archosaurian lineage, crocodilians present a unique opportunity to understand the evolutionary adaptations and relationships of the vertebrate immune system. Furthermore, crocodilians are important habitat modifiers,[4] economic commodities (i.e., tourism, hunting, and leather production[11]), and as they are long-lived top trophic carnivores, they may serve as indicators of environmental quality.[1,2] Consequently, crocodilians are logical study subjects to investigate how anthropogenic stressors may influence health of the species and may reflect ecosystem health.

Current crocodilian immunological work has been restricted to either studies investigating innate immunity (reviewed in Finger and Isberg[10]), *in vivo* and *ex vivo* assays,[12] or on the preliminary identification and characterization of leukocytes.[10,13,14] These and similar studies often rely on sampling individuals once, which, while common and even necessary in field studies, may increase risk of confounding variables that may skew results. The ecologic assessment value of some previously utilized techniques, such as phytohemmaglutinin injection, may also offer limited interpretive value due to the multiple factors influencing the resulting response and consequently, the biological interpretation.[12] The present lack of well-defined techniques whereby field-collected crocodilian blood can be analyzed for phenotype and functionality, greatly limits utility of blood for ecological assessments. While the development of assays to further investigate immunity is necessary, researchers also need to be able to obtain samples and then accurately process and assess these samples in a timely fashion to ensure no degradation of the sample has taken place. Most crocodilian research is conducted in more remote areas, distant from processing centers that have sophisticated equipment for analysis of samples, such as flow cytometry. Therefore, there is a need to understand the impact of post sampling time and handling on hematological and immune indices.

In this study, we evaluated blood samples collected from the American alligator (*Alligator mississippiensis*), a crocodilian species endemic to the southeastern United States and that has been at the forefront of crocodilian research. The impetus for this study was twofold. First, we wanted to determine the quantitative effects of time, post-blood sampling (e.g., 4 hr vs. 24 hr) on hematological and select immune indices, such as total red blood cells, packed cell volume, plasma total solids, total leukocytes, leukocyte subsets plus spontaneous, and mitogen-induced T cell proliferation, *in vitro*. Secondly, we wanted to initiate preliminary reference intervals of

hematological indices in juvenile American alligators as a tool to aid in assessing the health of wild or experimental specimens when they encounter anthropogenic activities.

EXPERIMENTAL

Animals

Juvenile American alligators, aged 2–3 years, were housed in three pens (dimensions of Pens 1, 2, and 3 were 3.6×4.59 m, 4.60×5.03 m, and 3.06×4.90 m, respectively), eight individuals per pen, in an enclosed aquatic animal facility, a climate-controlled (approximately 22.8°C on sampling days) greenhouse-type building, at the Savannah River Ecology Laboratory (SREL) in Aiken, SC, USA. Light:dark cycles were ambiently regulated via light filtration through glass panes on the roof and windows on the side of the building. Twenty of these animals were originally obtained one year prior to this experiment with another four obtained three months prior, at Rockefeller Wildlife Refuge in Grand Chenier, LA, USA. Each pen contained one main basking platform (Pens 1, 2, and 3 platforms were 1.04×1.78 m, 1.07×1.74 m, and 1.04×1.78 m, respectively) and multiple smaller concrete platforms (39.32×18.29×18.59 cm). Unheated (ambiently regulated) water (depths of Pens 1, 2, and 3 were 31.70 cm, 31.09 cm, and 36.58 cm, respectively) was continuously filtered at all times and alligators were fed Mazuri Crocodilian Diet (0062615; PMI Nutrition International LLC, Brentwood, MO, USA) three times weekly until satiation (i.e., alligators no longer interested in feeding). All alligator handling, husbandry, and blood collection procedures were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee.

Juvenile Alligator Identification, Body Size, and Sex

Following blood sampling of the juvenile alligators, head length (HL), total length (TL), and tail girth (TG) of each individual were measured with a tape measure as described by Isberg et al.[15] Alligators were sexed using blunt-nosed tweezers according to Allsteadt and Lang.[16] Scute cuts were used to identify individual alligators as described by Isberg et al.[17] for saltwater crocodiles, *Crocodylus porosus*.

Alligator Whole Blood Collection

Blood collection of the juvenile alligators occurred over a 2-week interval on two days, with sampling commencing at the same time period on both days (1000 hr). In the first collection, three alligators were captured

and 10 mL of blood were obtained per animal from the occipital sinus with a heparinized washed syringe (200 μL drawn into each syringe and subsequently expelled) within 5 min of capture. The blood was immediately transferred into sterile lithium heparin vacutainer tubes (Becton Dickinson, San Antonio, TX, USA), thoroughly mixed via gentle inversion 3–4 times, and stored on ice until arrival 4 hr later at the Immunotoxicology laboratory at the College of Veterinary Medicine at the University of Georgia in Athens, GA, a 2.5 hr drive from the SREL. Therefore, including capturing and bleeding multiple alligators, the first window of time for blood sample analyses was 4 hr post-collection. As mentioned in the introduction, this is a common situation when field sites and resident species are distant to equipped laboratories. For the second blood collection, seven alligators were sampled using the same experimental protocol outlined above.

Upon arrival, each tube of alligator blood was thoroughly mixed with a sterile 10 mL Pasteur pippete and split into 2 equal volumes. One volume of alligator blood was aliquoted back into the original heparin tube and labeled with the alligator number and a "4 hr" designation. This blood was then immediately processed for analysis. The second equivalent volume of heparinized blood was transferred to a sterile red top vacutainer tube (Becton Dickinson, San Antonio, TX, USA) and labeled by alligator number and a "24 hr" designation, then refrigerated at 7°C. Twenty-four hours post initial blood collection, the 24 hr alligator blood was removed from refrigeration, incubated at 23°C for 2 hr then subjected to the same analysis as the previous day's 4 hr alligator blood.

Alligator Peripheral Blood Leukocyte Enrichment

Prior to leukocyte enrichment, 100 μL of heparinized whole blood from each alligator tube was transferred into a separate 500 μL microfuge tube for blood hematology and whole blood cell enumeration. A "slow spin" protocol used in birds[18] was adapted to enrich for alligator leukocytes. Briefly, each alligator blood sample was centrifuged in an Eppendorf 5810R (15 amp, Hauppauge, NY) centrifuge at 40 xg, 10 min at 23°C with the acceleration and break set to zero. The tubes were carefully removed. Under aseptic technique, a sterile Pasteur pipette was inserted into the plasma to ~ 2 cm above the plasma buffy coat layer. The Pasteur pipette was moved in a clockwise direction to facilitate a swirl or "funnel-like" effect, which caused the leukocytes to resuspend into the plasma layer. The plasma was then carefully removed and transferred into a sterile 15 mL conical polystyrene centrifuge tube (Fisher Scientific, Waltham, MA, USA) containing 5 mL of incomplete RPMI media (Cellgro, Herndon, VA, USA). The tubes were centrifuged at 240 xg, 12°C for 10 min. The supernatant was discarded and the cells were resuspended in 5 mL of incomplete RPMI media (Cellgro, Herndon, VA,

USA) and centrifuged (240 xg, 7°C, 10 min). The supernatant was discarded and the enriched alligator cells were resuspended in 2 mL of complete RPMI media (10% adult alligator serum, L-glutamine, 1% non essential amino acids, 2% penicillin-streptomycin). A 100 μL aliquot from each tube was transferred into a 500 μL microfuge tube to enumerate cell recovery.

Based on previous leukocyte enrichment work with other non-mammalian species,[18,19] we would recommend working with whole blood volumes of not less than 5 mL. However, once the user masters the slow spin leukocyte enrichment technique, it is possible to enrich alligator lymphocytes with as little as 3 mL of alligator whole blood.

Alligator Whole Blood Smears

Approximately 10 μL of the peripheral blood were used to make blood smears for leukocyte differential counts. Once air-dried, the slides were stained with Wright Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) using a 10 min full stain/ 10 min diluted stain protocol. For each slide, 250 leukocytes were enumerated for a 5-point differential (lymphocyte, heterophil, monocyte, basophil, and eosinophil) and expressed as percent (%) using a Nikon Alphaphot-2/YS2 compound light microscope (Southern Micro Instruments, Marietta, GA, USA) under 100x magnification.

Packed Cell Volume and Total Solids

To assess alligator packed cell volume (PCV) and total solids, 75 mm microcapillary hematocrit tubes were filled to ~60 mm with whole blood, sealed with vinyl putty, and centrifuged at 14,489 xg, 3 min, 23°C, in a micro-hematocrit centrifuge, model C-MH30 (Unico, South Brunswick Township, NJ, USA). Each alligator PCV was read on a micro-capillary reader (International Eastern Company, Needham, MA, USA) and expressed as a percent (%). Using the plasma from the hematocrit tubes, alligator estimated total plasma solids values were measured with a TS 400 Handheld Refractometer (Reichert, Depew, NY, USA) and expressed as g/dL.

Alligator Whole Blood and Enriched Leukocyte Enumeration

A 10 μL aliquot of whole blood from each alligator was serially diluted 1:1000 in Ca free/Mg free PBS (Cellgro, Herndon, VA, USA). A 20 μL aliquot was loaded into a SD100 Nexcelom cell counting chamber and the alligator whole blood was enumerated using a Nexcelom Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, USA). The values were reported as $\times 10^6/\mu L$. For enumeration of the enriched leukocytes, a

1:2 dilution of the cells in Trypan Blue Solution 0.4% (w/v) in normal saline (Cellgro, Herndon, VA, USA) was made prior to loading 20 μL into a SD100 Nexcelom cell counting chamber. Viability and recovery were determined and the cells were diluted to 2.5×10^6 cells/mL and stored at 7°C for the cell proliferation assays and cytospin preparation.

Evaluation of Spontaneous and ConA-induced Leukocyte Proliferation

On the day of the experiment, select concentrations of Concanavalin A (ConA; 0.1, 1.0, 10, and 100 μg/mL) were diluted in fresh complete RPMI (Roswell Park Memorial Institute) media consisting of 10% adult alligator serum, 2% penicillin-streptomycin, and 1% nonessential amino acids in Ca+ and Mg+ free RPMI-1640 media supplemented with L-glutamine (Cellgro, Herndon, VA, USA). Duplicate sets of 96 well tissue culture plates (Corning, Corning, NY, USA) received 100 μL per well of media alone or ConA at the above concentrations. One set of plates was labeled "4 hr" and the other labeled "24 hr". The 4 hr plates were sealed in parafilm and stored at 7°C. The 24 hr plates were sealed in parafilm and stored at −25°C. For the 4 hr proliferation assay, the "4 hr" plates were removed from refrigeration and 100 μL of enriched leukocyte cells were pipetted to quadruplicate wells containing 100 μL of media alone or ConA at the above concentrations. On the day of the 24 hr experiment, the "24 hr" plates were removed from the −25°C and placed in the 7°C for 2 hr to acclimate to the same temperature; the cell pipette procedure was identical to the 4 hr experiment. Each set of plates (4 and 24 hr) was cultured at 30°C, 5%CO_2 in a humidified tissue culture incubator for 64 hr. The plates were then temporarily removed from the incubator and each well received 20 μL of the alamarBlue™ dye (Thermo Fisher Scientific, Oakwood Village, OH, USA) and then returned to the incubator for an additional 8 hr.[19,20] The plates were removed and the cells evaluated for both degree of spontaneous and ConA-induced proliferation measuring the change in fluorescence of the cells using a Synergy4 microplate reader (Biotek, Winooski, VT, USA). The values were expressed as mean fluorescence ± SEM of the 4 replicate wells/ stimulant.

Peripheral Blood Leukocyte Enrichment Assessed by Cytospin

Lymphocyte purity post "slow spin" separation was determined by adding a 50 μL (~1.25×10^5 cells) aliquot of the peripheral leukocyte-enriched recoveries into individual cytospin slide chambers. The chambers were diluted with 150 μL of Ca free/Mg free PBS (Cellgro, Herndon, VA, USA) to reach the required 200 μL volume. The slide chambers were then capped

and centrifuged at 34 xg for 3 min at 23°C on a 7150 Hematology Slide-Stainer Cytocentrifuge (Wescor, Logan, UT, USA). All cytospin prepared slides were stained with Wright-Giemsa (Sigma-Aldrich, St. Louis, MO, USA) and evaluated on a compound light microscope (described above) under 100x magnification. For each slide, 250 leukocytes across a minimum of 10 fields were enumerated to determine the lymphocyte purity percentage. The values were expressed as mean % ± SEM of 10 alligator samples across two experiments.

Collection of Sub-Adult Alligator Serum

Prior to the study, sub-adult female (≈1.6m) American alligators, housed at Rockefeller Wildlife Refuge in Grand Chenier, LA, were captured, restrained, and their blood sampled from the post-occipital venous sinus with a heparinized washed-syringe to obtain serum, for later use in the juvenile alligator study, following procedures recommended by the Louisiana Department of Wildlife and Fisheries and Louisiana State University School of Veterinary Medicine. Following sampling, blood was placed aseptically into sterile 50 mL conical tubes (Fisher Scientific, Waltham, MA, USA) allowed to clot, chilled, and shipped to the University of Georgia College of Veterinary Medicine in Athens, GA. Subsequently, blood was centrifuged at 2292 xg for 10 min and serum was collected from each tube and pooled. The pooled adult alligator sera were heat inactivated for 30 min at 56°C in a water bath, filter sterilized, and aliquoted into sterile 50 mL conical tubes (Fisher Scientific, Waltham, MA, USA) and frozen at −20°C.

Statistics

Statistical analysis was performed on the 4 hr and 24 hr data to assess the impact of sample processing time on the alligator blood parameters and leukocyte cell proliferation. The data were analyzed using an unpaired *t*-test and were sampled from Gaussian distribution and a two-tailed *P* value running the InStat 3 software for Macintosh by GraphPad Software. All data were tested for normality using the Kolmogorov-Smirnov test. Significance was set at $P \leq 0.05$.

RESULTS

Juvenile Alligator Identification, Body Size, and Sex

Mean (± SE) HL, TL, and TG for alligators in this study was 11.95 ± 0.23, 98.19 ± 0.96, and 1.32 ± 0.42, respectively. All individuals, except for two, were identified as female.

Alligator Blood Cellularity and Estimated Total Plasma Solids Values are Stable for 24 hr

Analysis of total red blood cell count, packed cell volume, mean corpuscular volume and estimated total solids from heparinized blood samples of 10 alligators yielded results that were comparable at 4 hr and 24 hr (Table 1). Evaluation of the 5-pt differential from blood smears (Figure 1) made at 4 hr and 24 hr post blood collection also yielded values that were numerically similar (Table 1). Analysis of each of the nine hematologic values across the 10 alligator samples showed no significant difference across the two time points.

Alligator Enriched Leukocyte Recovery and Viability are Constant up to 24 hr

Slow spin separation, a cell-sparing procedure, yielded numerically similar leukocyte recoveries and viabilities at 4 hr and 24 hr time points that were not significantly different (Table 2). The average enriched leukocyte recovery per milliliter of alligator whole blood at 4 hr and 24 hr was 5.8×10^6 and 5.4×10^6, respectively. Cytospin evaluation of these enriched leukocyte fractions (Figure 1) showed that the majority ($\geq$90 %) were lymphocytes (Table 2). Analysis of these data from the 10 alligators across both time points also confirmed that the yields were not significantly different.

Alligator Spontaneous and ConA-Induced Lymphocyte Proliferation is Comparable at 4 hr and 24 hr

Alligator lymphocytes from 4hr and 24 hr enrichments when cultured *in vitro*, had comparable spontaneous proliferation. The spontaneous proliferation of the alligator lymphocytes at 4 hr and 24 hr were 3268 $\pm$ 58.4 and 2894.4 $\pm$ 75.0 (mean fluorescence $\pm$ SEM), respectively. ConA-induced proliferation yielded the same pattern of response showing a measureable increase at 10 and 100 μg/mL (1 and 10 μg/well) (Figure 2).

DISCUSSION AND CONCLUSION

The results of our study clearly show that with proper collection and storage, alligator hematological, and select immune parameters are stable and the lymphocytes are functional at least up to 24 hr post blood collection. In a previous study with horses, we observed a similar outcome with peripheral blood except the blood was stored at 23°C. However, the % of neutrophils declined at 24 hr.[21] This was not observed in the present study and may be attributed to the chilling of the blood (7°C), which is a common cell

TABLE 1 The Effect of Time Post Blood Collection on Alligator Hematology, Total Solids, and Peripheral Blood Leukocytes

	Alligator Hematology and Total Solids				Peripheral Blood Leukocyte Differential				
Time (hr)	Total RBC Recovery (Mean $\times 10^5/\mu L$ ± SEM)	PCV (Mean % ± SEM)	MCV (Mean fL ± SEM)	Total Solids (Mean g/dL ± SEM)	Lymphocytes (Mean % ± SEM)	Heterophils (Mean % ± SEM)	Basophils (Mean % ± SEM)	Eosinophils (Mean % ± SEM)	Monocytes (Mean % ± SEM)
4	3.5 ± 0.2	25.0 ± 0.4	734.9 ± 50.4	5.3 ± 0.1	58.4 ± 1.3	23.0 ± 0.8	9.4 ± 0.8	0.8 ± 0.2	8.6 ± 0.9
24	3.4 ± 0.2	27.5 ± 0.7	843.7 ± 56.8	5.1 ± 0.1	60.3 ± 1.7	23.2 ± 1.0	7.7 ± 0.9	0.6 ± 0.2	8.2 ± 0.7

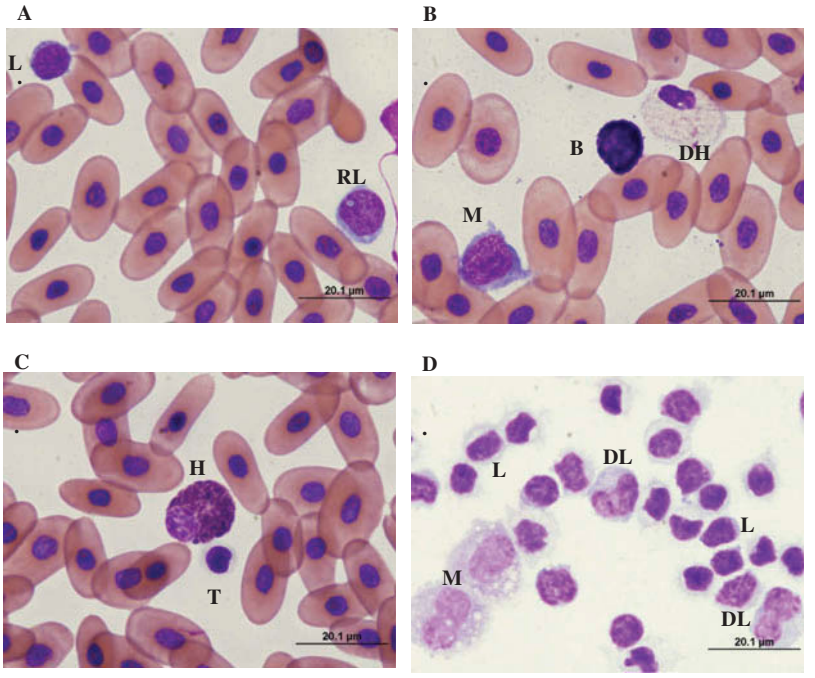

FIGURE 1 Representative images of alligator blood smears (**A-C**) and an enriched leukocyte cytospin (**D**). For the blood smears, examples of a lymphocyte (L), reactive lymphocyte (RL), monocyte (M), basophil (B), heterophil (H), degranulated heterophil (DH), and a thrombocyte (T) are provided. For the cytospin, the majority of the cells were lymphocytes (L). Lymphocytes undergoing cell division (DL) and monocytes (M) were also present.

TABLE 2 The Effect of Time Post Blood Collection on Enriched Leukocyte Fraction Recovery and Viability

	Total Recovery and Viability		Enriched Leukocyte Fraction from Total Blood				
Time (hr)	Total Recovery ($\times 10^7$) (Mean ± SEM)	Viability (Mean % ± SEM)	Lymphocytes (Mean % ± SEM)	Monocytes (Mean % ± SEM)	Heterophils (Mean % ± SEM)	Basophils (Mean % ± SEM)	Eosinophils (Mean % ± SEM)
4	2.9 ± 0.5	92.4 ± 2.1	90.8 ± 0.8	9.1 ± 0.8	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
24	2.7 ± 0.3	94.2 ± 0.5	90.0 ± 1.3	9.4 ± 1.3	0.4 ± 0.2	0.2 ± 0.1	0.0 ± 0.0

culture technique to decrease cell attachment to glass and plastic surfaces. Use of this cold storage technique worked well with tht alligator blood cells since all the measured hematologic parameters as well as the viability of the enriched lymphocytes (>90%) were comparable across the two time points.

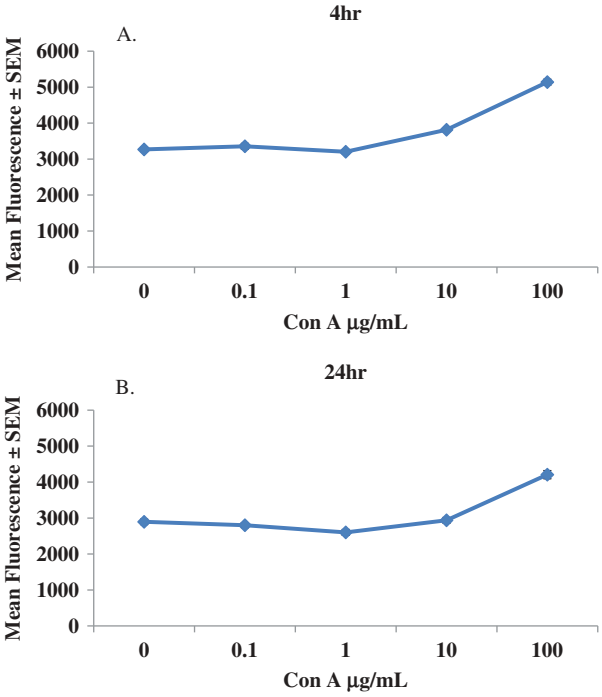

FIGURE 2 ConA peripheral blood lymphocytes proliferation curves from 10 alligators were cultured for 72 hr. Alligator peripheral blood lymphocytes processed at 4 hr (**A**) and at 24 hr (**B**) post blood collection were each cultured at 2.5×10^5 cells/100 µL/well with 100 µL of ConA (0, 0.1, 1,10, 100 µg/mL) for 64 hr. AlamarBlue™ (20 µL) was then added to each well and incubated an additional 8 hr. Values are reported as mean fluorescence ± SEM.

There were two additional observations worth noting regarding the alligator lymphocytes in culture. First, there was a slight decrease in the fluorescent response in the 24 hr plates when compared to the samples in the 4hr plates. We attributed this to the cold storage temperature (−25°C) of the pre aliquoted media and ConA in the 24 hr culture plates prior to their use. In previous bird studies, cold storage at −40°C had little impact on media and ConA integrity in culture.[22] Since a −40°C freezer was not available for this study, a temperature lower than −25°C is required to prevent a slight degradation in media and ConA. Regardless, the growth curves were comparable across the two time points providing the same level of interpretation. A second observation was made regarding the concentration of the alligator lymphocytes that were cultured and their response to the ConA. In most mammalian species such as mice, the typical lymphocyte cell culture concentration is 5.0×10^5/well.[20] Using a concentration of 2.5×10^5 lymphocytes/well, based on previous work with quail and chickens, the alligator lymphocytes yielded a ConA dose response curve different from

that seen in rodents. The highest ConA concentration of 100 μg/mL (10 μg/well) is toxic to mouse lymphocytes, but instead continued to stimulate the alligator lymphocytes suggesting a higher tolerance to this plant lectin.

Alligator hematology and total solids values presented herein are similar to those presented in other studies.[23,24] Likewise, leukocyte differential values presented here are similar to those observed in uninfected wild alligators[23] and in other species,[14] with a greater number of lymphocytes in peripheral blood than heterophils. In contrast, Mateo et al.[24] detailed higher levels of heterophils (54.7%) relative to lymphocytes (23.9) in 8–10 month old alligators. However, such a result of increased heterophils relative to lymphocytes observed in that study may have been related to infection.[23]

One potential caveat of this study was the use of heparinized washed syringes to obtain blood from juvenile alligators. Often necessary to prevent clot formation during a prolonged collection, the use of anticoagulants in collection may skew results, especially of small volumes,[25] as it is often difficult to maintain the necessary ratio of blood to anticoagulant.[26,27] Use of heparin, in particular, as an anticoagulant during blood collection may also affect staining of leukocytes or contribute to clumping, which may consequently affect cell counts.[27] However in that study, as well as in a study examining PCV and TS in juvenile alligators,[25] the investigators used 0.2 mL of whole blood, a minimal volume required for commercial diagnostic laboratories. In the present study, we collected 10 mL, which were then transferred into heparinized glass vacutainer tubes. This amount of blood was well within the acceptable heparin to blood ratio (10–30 USP units/mL of blood) and as a result none of problems reported in the study by Strik et al.,[27] were observed in the present study.

Our findings demonstrate that blood samples obtained from crocodilians in the field can still be utilized 24 hr later to investigate different aspects of hematologic parameters and non-specific immune function. In addition, using the slow spin separation procedure established in birds, one can easily and consistently isolate alligator lymphocytes from whole blood and obtain a purity of $> 90\%$ with a viability of $> 92\%$ in juvenile alligators to study as various aspects of alligator immunity. However, these yields may change based on the age or sex of the alligators, season and other variables.[10]

In summary, we showed that with proper collection and storage, alligator blood can be analyzed within 24 hr of collection and still yield consistent hematologic and immune endpoints, which can potentially aid crocodilian researchers to assess the health status of this important ecological top-tier species.

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