Title: Peripheral blood hematology, plasma biochemistry, and the optimization of an *in vitro* immune-based assay in the brown watersnake (*Nerodia taxispilota*)

Abstract

Reptiles represent a phylogenetic lineage that provides a unique link between ectothermic anamniotes and endothermic amniotes. Compared to mammalian and avian species, our understanding of the reptilian immune system is greatly lacking. This gap in knowledge is largely due to an absence of established immune-based assays or specific reagents for these species. In the present study, brown watersnakes (*Nerodia taxispilota*) were live-captured in the wild, sexed, weighed, measured, bled via the caudal vein and released. At 24 hr post-collection, peripheral blood leukocytes were enriched and evaluated with an established mammalian *in-vitro* lymphocyte proliferation assay. Snake peripheral blood leukocyte enrichment yielded > 90% lymphocytes with viabilities averaging 81.5%. Baseline physiologic data for *N. taxispilota*, including hematology and total solids, leukocyte differentials, cell recovery and plasma biochemistry, were also collected. Cells cultured with Concanavalin A exhibited significantly increased proliferation at both 72 and 96 hr. These preliminary results show that enriched peripheral blood from wild-caught *N. taxispilota* provides a sufficient yield of leukocytes that can be cultured and functionally evaluated using a standard mammalian *in-vitro* immune-based assay.

Keywords: snake, peripheral blood collection, hematology, lymphocyte separation, mitogen, proliferation

Introduction

Assessing an animal's capacity to withstand foreign antigenic assault (*i.e.*, pathogens, toxins, toxicants) is a fundamental component of ecoimmunological studies. Snakes and other reptiles are unique in that they are ectothermic amniotes, bridging the gap between ectothermic fishes and amphibians to endothermic birds and mammals. As such, reptiles present an opportunity to provide unique insights into the evolution of the vertebrate immune response.[1] Snakes represent a group of reptiles comprised of more than 3,000 species, and thus inhabit a wide diversity of environments.[2] Furthermore, snakes are important predators within their respective ecosystems and can have relatively long-life spans with a longer potential exposure to environmental contaminants.[3,4] Collectively, these traits suggest that snakes may be useful bioindicators for anthropogenic impacts on ecosystem health.[5]

Establishing a reliable panel of immune assays would provide researchers with the necessary tools to study reptile immunity as well as assess the effects of anthropogenic stressors on reptilian health.[6] The snake's immune system is similar to other vertebrates in that they possess an innate, cell-mediated, and humoral immunity. Relative to mammalian and avian species, there is still a paucity of data available regarding reptilian immunity – especially in snakes.[1] While interest in the snake immune system has increased in recent years, the majority of studies involving snake immunity have been limited to the examination of hematological characteristics, innate immunity, and the characterization of leukocytes.[7-10] Unfortunately, these techniques do not provide an assessment of immune cell phenotype and functionality.[11] Thus, establishing and validating commonly used mammalian *in vitro* immune assays in snakes will allow for a more thorough examination of anthropogenic effects on snake immunity.

This study optimized a select panel of established *in vitro* mammalian immune-based assays, while generating baseline hematological data for the brown watersnake (*Nerodia*

taxispilota), a common, large-bodied riverine snake species found in the southeastern United States. The immune panel evaluated total leukocytes, performed white blood cell differentials, and measured spontaneous and mitogen-induced T cell proliferation, *in vitro*. In addition, baseline hematological parameters, including total red blood cell counts, packed cell volume, and plasma total solids were collected as well as reference plasma biochemistry data for this snake species.

Methods

Animal and Blood Collection

Snakes (N = 12) were collected by hand from the Savannah River near Augusta, Georgia, USA during late spring (5 May) of 2019. Each snake was sexed, measured (snout-vent length [SVL] and tail length [TL] to nearest 1 mm) and bled via the caudal vein. All snakes were immediately released following processing. Snake captures started in the morning (0930 hr) and were finished by the evening (1830 hr). For each snake, 3 – 5.5 mL of blood were obtained (≤ 1% of the animal's body mass) within 3 – 5 min of capture. Peripheral blood samples were collected from the caudal vein using aseptic technique. Blood samples were collected with a 23 g needle and then immediately transferred to sterile sodium heparin vacutainer tubes (Becton Dickinson, San Antonio, TX, USA) with a 18 g needle. Once in vacutainer tubes, samples were mixed via gentle inversion 3 – 5 x and stored at approximately 20°C until arrival at the College of Veterinary Medicine at the University of Georgia (Athens, GA) the next morning (0800 hr). A small aliquot of fresh whole blood (non-heparinized) was taken from the initial blood collection (post vacutainer transfer) to make whole blood smears and evaluate packed cell volume (PCV).

All samples for this experiment were collected, transported, and processed within 24 hr of the initial capture and bleeding.

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Whole Blood Smears

On the day of blood collection, 10 μL of whole blood were used to make blood smears for leukocyte differential counts. Smears were allowed to air-dry overnight and then stained with Wright Giemsa (Sigma Aldrich, St. Louis, MO, USA) using a 10-min full stain/10-min diluted stain protocol. A total of 200 leukocytes per slide were enumerated for a 5-point differential (azurophil, basophil, heterophil, lymphocyte, and monocyte) in the monolayer of the smear. Cell counts are expressed per microliter and as percentages (%). The white blood cell (WBC) estimate was calculated by multiplying the average number of WBCs in 10 microscope fields by objective power squared.[12] All slides were counted using an Olympus CX31 compound light microscope (Olympus America Inc., Center Valley, PA) under 40x magnification. Recent literature in reptilian hematology was used to guide identification and enumeration of cell types, and a board-certified clinical pathologist (KM) supervised the enumeration of whole blood smears.[13-15]

Packed Cell Volume and Total Solids

A small aliquot of whole blood was used to determine PCV and total solids. Briefly, microhematocrit tubes were filled with whole blood, sealed with Chā-seal tube sealing compound (Chase Instruments Co., West Babylon, NY, USA), and centrifuged at 14,489 x *g* for 3 min at ~ 23°C in a microhematocrit centrifuge (Unico, South Brunswick Township, NJ, USA) to evaluate PCV. Snake PCV was read using a microcapillary reader (International Eastern

Company, Needham, MA, USA) and expressed as a percent (%). Total solids (g/dL) were evaluated using a TS 400 Handheld Refractometer (Reichert Technologies, Depew, NY, USA).

Peripheral Blood Leukocyte Isolation and Enrichment

A "slow spin" separation protocol previously used in birds and crocodilians was used to isolate snake leukocytes.[16,17] All peripheral blood samples less than 4 mL in volume received an initial addition of Ca free/Mg free PBS (1 – 3 mL depending on initial volume to a total of 6 mL; Fisher Scientific, Ottawa, Ontario). Blood samples were centrifuged in an Eppendorf 5810R centrifuge (15 amp, Hauppauge, NY) at 40 x *g* and 23°C for 15 min with the acceleration and brake set to zero. A minimum of 0.5 mL of plasma was collected from each blood sample and stored at -80°C until biochemistry analysis. Using aseptic technique in a laminar flow hood, a sterile Pasteur pipette was inserted approximately ~ 2 cm above the plasma buffy coat layer. The Pasteur pipette was then moved in a clockwise direction until the leukocytes were separated and suspended into the plasma layer. The plasma-buffy coat layer was aseptically removed and transferred into a sterile 15 mL conical polystyrene centrifuge tube (Thermo Fisher Scientific, Waltham, MA, USA) containing 5 mL of complete RPMI media (10% fetal bovine serum, L-glutamine, 1% non-essential amino acids, 2% penicillin-streptomycin). The enriched cell suspensions (containing resuspended leukocytes) were centrifuged at 240 x *g*, 7°C for 10 min. The cells were then resuspended in 5 mL complete media. To maximize snake leukocyte enrichment, the vacutainer tubes (containing the original snake whole blood samples) were then gently resuspended with 5 mL room temperature PBS and centrifuged at 40 x *g* and 23°C for 15 min. The second buffy coat cell collection consisting of the plasma-PBS-buffy coat layer was aseptically removed and transferred into the sterile 15 mL conical polystyrene centrifuge tube

(Fisher Scientific) containing the first collection of enriched cells in 5 mL of complete RPMI media. These combined enriched cell suspensions were centrifuged at 240 x *g*, 7°C for 10 min, resuspended in 2 mL of complete RPMI media, and then enumerated.

Whole Blood and Enriched Leukocyte Enumeration

A 10 µL aliquot of whole blood from each snake was serially diluted 1:1000 in Ca free/Mg free PBS (Fisher Scientific, Ottawa, Ontario). Next, a 20 µL aliquot of each diluted sample was loaded into a SD100 Nexcelom cell counting chamber and snake whole blood was enumerated using a Nexcelom Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, USA). To enumerate enriched leukocytes, 100 µL was collected from the enriched cells in 2 mL of complete media (Fisher Scientific, Ottawa, Ontario) and transferred into 500 µL microfuge tubes containing a Trypan Blue Solution, to assess viability. A 20 µL of each diluted cell suspension was loaded into SD100 Nexcelom cell counting chamber slide, enumerated, and assessed for viability. Snake whole blood cell and enriched leukocyte values were reported as N x 10^6/mL.

Peripheral Blood Leukocyte Enrichment Assessment via Cytospin

Purity of lymphocytes was assessed by adding a 20 µL (~ 5 x 10^4 cells) aliquot of peripheral leukocytes into individual cytospin slide chambers. Chambers were diluted with 180 µL of Ca free/Mg free PBS (Fisher Scientific, Ottawa, Ontario) to a total volume of 200 µL. Slide chambers were centrifuged at 34 x *g* for 3 min at 23°C using a 7150 Hematology Slide-Stainer Cytocentrifuge (Wescor, Logan, UT, USA). Slides were then stained with Wright-Giemsa (Sigma-Aldrich, St. Louis, MO, USA) using a 10-minute full stain/10-minute diluted

stain protocol. Stained slides were evaluated on an Olympus CX31 compound light microscope (Olympus America Inc., Center Valley, PA) under 40x magnification. A total of 200 leukocytes across a minimum of 10 fields were enumerated to determine lymphocyte purity percentage. Values are expressed as mean % ± 1 SE. A board-certified clinical pathologist (KM) supervised the enumeration of snake cytospin smears.

Evaluation of Spontaneous, Con A, and LPS-induced Leukocyte Proliferation

On the day that samples were processed in the lab, 100 µL/ well of Concanavalin A (Con A; 1.0, 10, 50, and 100 µg/mL) and lipopolysaccharide (LPS; 5 and 50 µg/mL) in complete RPMI-1640 media were aliquoted in 96 well tissue culture plates (Corning, Corning, NY, USA). Each well received 100 µL of media alone, Con A, or LPS at the concentrations mentioned above. To each well, 100 µL of enriched leukocytes (4 x 10^6 cells/mL) were pipetted in triplicate. All plates were cultured at 30°C, 5% CO_2 in a humidified tissue culture incubator for 48 or 72 hr. At 48 or 72 hr, plates were temporarily removed from the incubator and 20 µL of alamarBlue™ dye (Thermo Fisher Scientific) were added to each well. The alamarBlue™ dye is a water-soluble dye that when initially added to cell cultures is in an oxidized state (*i.e.,* blue color). Cellular proliferation increases the metabolic activity of the cells which chemically reduces the dye in the media, changing the color of the dye from blue to red.[18] This dye also fluoresces when reduced, which is an added benefit to researchers using a fluorescent detection platform. Plates were then returned to the incubator for another 24 hr. Plates were evaluated for spontaneous (media + cells only) and Con A or LPS-induced proliferation. The degree of proliferation was assessed by measuring the absorbance of cells at 570 and 600 nm via a

Synergy4 microplate reader (BioTek, Winooski, VT, USA). Values are expressed as mean delta of optical density (ΔOD570/600) ± 1 SE of the wells (triplicate).

Plasma Biochemistry

Plasma samples collected from whole blood were analyzed using the Avian-Reptile Profile Plus rotor in a VetScan VS2 (Abaxis Inc., Union City, California, USA). These rotors provide information for 12 biochemistry analytes, including albumin (Ab), aspartate aminotransferase (AST), bile acids (BA), calcium (CA^{++}), creatine kinase (CK), globulin (GLOB), glucose (GLU), potassium (K^{+}), sodium (Na^{+}), phosphorous (PHOS), total protein (TP), and uric acid (UA). Frozen banked samples were thawed in a refrigerator and processed the same day. The rotor was loaded with 0.1 mL of plasma and analyzed per the manufacturer's instructions.

Statistics

Statistical analyses were performed on the cell proliferation data to assess the best concentrations of mitogens (Con A and LPS) to measure proliferative responses (ΔOD570/600) for immunoassays in *N. taxispilota*. These data were analyzed using ANOVAs. Data were evaluated for normality and homogeneity of variances. A separate ANOVA was used for each incubation period (72 and 96 hr) for each mitogen group (media alone vs. multiple concentrations of a single mitogen). Differences among groups were considered significant with a $p < 0.05$. A post hoc analysis was performed (Tukey's HSD) if significant differences were found among groups. All analyses were performed in RStudio.[19]

Results

Snake Body Size and Sex

Twelve adult *N. taxispilota* (7 females, 5 males) were sampled for this experiment. Mean (± 1 SE) SVL and TL of females was 865.9 ± 18.5 mm and 254.3 ± 9.2 mm, respectively. Mean SVL and TL of males was 764.2 ± 21.8 mm and 260.4 ± 8.7 mm, respectively. Previous studies in this species suggested that female and male brown watersnakes near the Savannah River Site (Aiken, SC, USA) are sexually mature at 795 mm (~ 5-6 years of age) and 500 mm (~ 3 years of age) snout-vent length (SVL), respectively.[20,21] All of the males sampled in this study were well above the 500 mm threshold (range 711 – 816 mm SVL). All females, with the exception of one individual at 786 mm, were above the 795 mm threshold (range 786 – 922 mm SVL). Based on the smallest female's size at capture and data from previous studies, we postulated that she was sexually mature.[20,21]

Snake Hematology and Total Solids

Analysis of total red blood cell (RBC) cellularity, PCV, mean corpuscular volume and total solids revealed that the range in numeric values for the 12 snakes were relatively tight. The standard errors for each of the four blood biomarkers were less than 10% of the mean (**Table 1**). One individual was not included in the dataset for hematology and total solids due to suspected lymph contamination.

Snake Leukocyte Differentials and Calculated Cellularity

205 Analysis of brown watersnake whole blood smear leukocyte differentials revealed that
206 lymphocytes were the most common cell type, followed by azurophils, heterophils, basophils
207 and then monocytes (**Table 2**).
208
209 ***Total Leukocyte Recovery and Purity***
210 Average enriched leukocyte recovery per snake was 4.8 x 10^6/mL. Analysis of the
211 cytospin slides confirmed that the enriched leukocytes recovered were > 90% lymphocytes and
212 average viability was 81.5% (**Table 3**).
213
214 ***Effects of Mitogens on Snake Lymphocyte Proliferation***
215 Snake leukocytes cultured with LPS (5 and 50 μg/mL) did not exhibit significantly
216 increased lymphocyte proliferation at either 72 or 96 hr (**Figure 2**). LPS-induced proliferation
217 was comparable to spontaneous proliferation at 72 and 96 hr ($F_{2,33}$ = 0.18 – 1.97, p = 0.156 –
218 0.838). Con A-induced proliferation exhibited a significant effect of exposure ($F_{4,55}$ = 7.7 –
219 11.39, both $p < 0.001$) concentration on snake lymphocytes, with similar trends observed at 72
220 and 96 hr (**Figures 3A and 3B**). At both 72 and 96 hr, ConA-induced proliferation at a
221 concentration of 50 μg/mL was significantly increased compared to spontaneous proliferation at
222 $p \leq 0.001$ and $p = 0.002$, respectively.
223
224 ***Snake Plasma Biochemistry***
225 Plasma biochemistry was evaluated on 9 of the 12 snakes. One snake had a low initial
226 blood volume, one sample was contaminated with lymph fluid post collection and the third

sample was diluted with PBS prior to collection. Of the 12 plasma biochemical analytes examined, only the bile acids and creatine kinase concentrations had wide ranges (**Table 4**).

Discussion

This study sought to determine whether snake peripheral blood could be aseptically enriched to yield an adequate lymphocyte cell number for use with an established mammalian *in vitro* immunoassay, while generating novel reference hematological and plasma biochemistry data for wild-caught brown watersnakes (*Nerodia taxispilota*).

Aseptically enriched *N. taxispilota* lymphocytes exposed to Con A successfully proliferated at both 72 and 96 hr, with the 72 hr culture period demonstrating the optimal peak in the Con A-stimulated growth curve. This proliferative response would appear to suggest that a large portion of the peripheral blood enriched lymphocytes were T cells, which has been reported in other species.[22-24] Exposure to LPS did not induce a significant increase in proliferation in *N. taxispilota* at 72 or 96 hr. Our results are similar to a recent study in a watersnake species from India (*Natrix piscator*), in which LPS-induced splenocyte proliferation (20 μg/mL of LPS) did not significantly differ when compared to spontaneous proliferation.[25] Since LPS is a potent inducer of B cells, it is possible that these cell types were absent or numerically too low in the enriched leukocyte fraction to induce a detectible proliferative response. Further, immune responses of reptiles are known to be strongly affected by the season.[26,27] Therefore, it is also possible that the *N. taxispilota* were sampled at a seasonal period when the B cells were less responsive.[27] Interestingly, other studies in snakes and turtles have reported that the LPS did cause a significant increase in lymphocyte proliferation.[8,28,29] A review of these studies revealed that the proliferation assay methodologies were different (e.g., basal cell concentration,

collection/extraction method, mitogen concentration, length of incubation), which could have impacted lymphocyte purity and/or function. In the present study, the peak Con A-stimulated proliferation was reached at 50 μg/mL or 5.0 μg/well cultured with 4.0 x 10^5 cells/well. Although the highest concentration of Con A (100 μg/mL or 10 μg/well) used in this study appeared to show signs of toxicity in *N. taxispilota* (demonstrated by a decline in proliferation), the toxic response in these snakes did not appear to be as dramatic as those seen in lymphocytes from other taxa.[16,30]

To our knowledge, this is the first study to provide preliminary reference data for blood hematology or plasma biochemistry in wild *N. taxispilota*. Snake plasma biochemistry and hematology values in our study were similar to the limited data available from a closely related snake species (*Thamnophis* spp.).[7] Leukocyte differentials were also comparable to those reported in other snake species, with lymphocytes (51.4%) being the most common and azurophils (35.5%) the second most common leukocyte type observed in *N. taxispilota* smears.[13] Heterophils (7.2%), basophils (4.2%), and monocytes (1.7%) all comprised a much smaller average portion of the leukocyte differential. It is important to consider the potential effects of handling stress on the reference blood hematology and plasma biochemistry of *N. taxispilota*. Capture and manual restraint of animals for blood collection is known to increase hormones that may alter results. A study in sea turtles showed that while WBC counts do increase significantly between 0 and 10 min of capture, the differences between 0 and 6 min were negligible.[31] Another study in garter snakes (*Thamnophis sirtalis*) found that bleed times of 10 min or less did not affect baseline corticosterone concentrations – a hormone significantly elevated during the stress response.[32] All snakes in this study were hand-captured and bled within 3 – 5 minutes, thus, we suspect that our values likely reflect baseline conditions.

In summary, the results of our study show that the collection, transport, and enrichment of blood leukocytes from wild *N. taxispilota* yields appreciable concentrations of lymphocytes, allowing for the assessment of this species' immune status. Similar to a previous study in alligators, lymphocytes from our snakes were successfully enriched within 24 hr, obtaining an average purity of >90%, which suggests that overnight field collections and subsequent immunoassays can be performed with ease.[17] Con A-induced proliferation yielded robust results, with best responses observed at a concentration of 50 µg/mL and 72 hr incubation period. Future studies should determine the optimal exposure concentration for LPS-induced proliferation in this species as well as other mitogens (*i.e.*, phytohemagglutinin [PHA] or pokeweed mitogen [PWM]). A previous study found that peripheral blood leukocytes from turtles responded differently to LPS relative to PWM.[29] Thus, future work should focus on other mitogens and other lymphoid tissues to evaluate B cell response in *N. taxispilota*. Once these techniques are optimized, researchers should be able to employ a suite of assays to evaluate both T and B cell function in *N. taxispilota*. In conclusion, using the immune methods developed and the hematologic and biochemistry data generated in this study, researchers working with this species should be able to assess inherent differences (e.g., reproductive impacts, seasonal effects, etc.) in the snake's health status as well as begin to understand how anthropogenic stressors impact their immune system.

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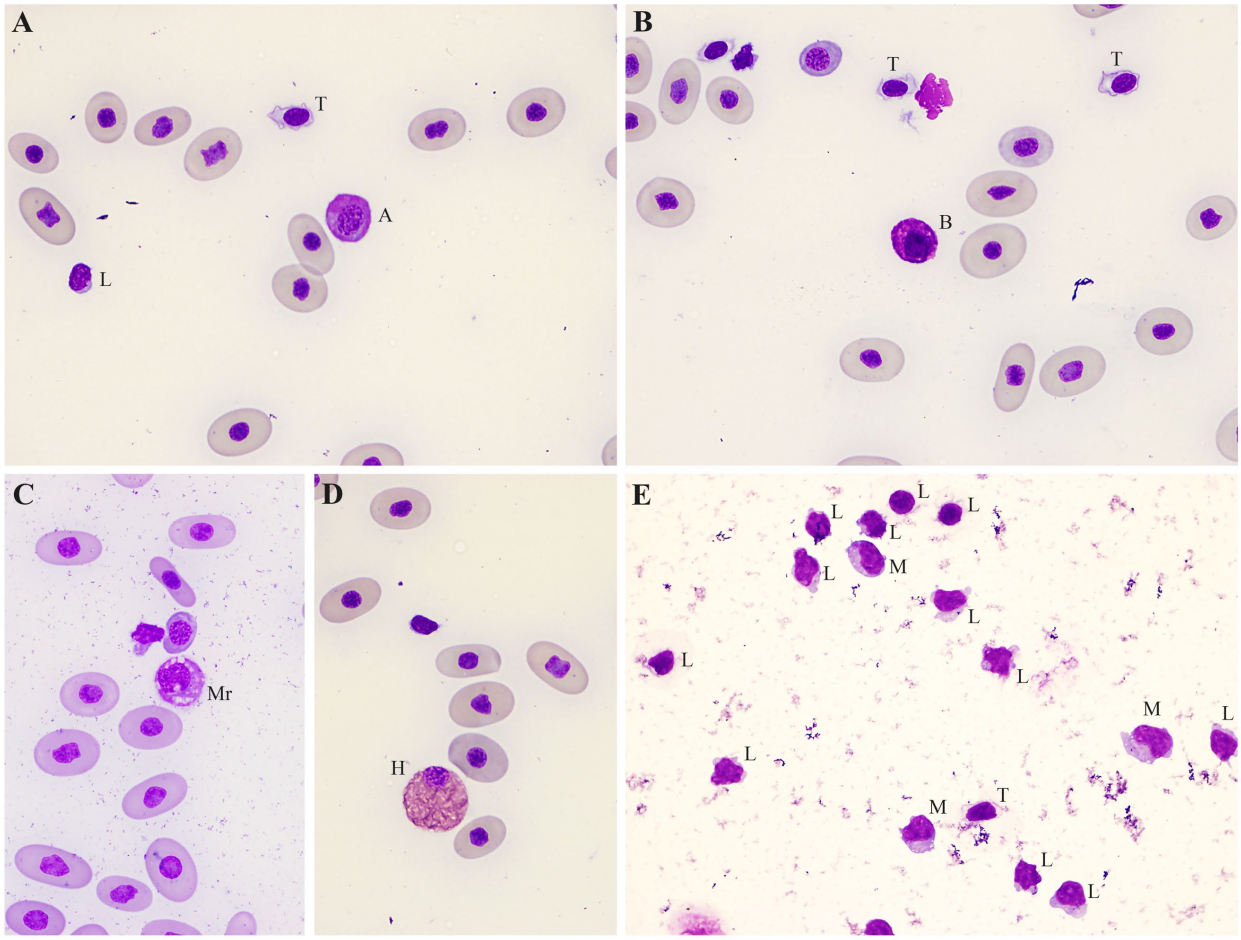

Figure 1: Images taken from brown watersnake *Nerodia taxispilota*) blood smears (panels A-D) and an enriched leukocyte cytospin (panel E). Examples of azurophils (A), basophils (B), heterophils (H), lymphocytes (L), monocytes (M), and reactive monocyte (Mr) are pictured. Examples of thrombocytes (T) are also provided.