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RESEARCH ARTICLE

Ultraviolet Radiation on Innate Immunity and Growth of Broad-Snouted Caiman (*Caiman latirostris*): Implications for Facilities Design

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Sunlight is a key environmental factor in almost all ecosystems, and it is necessary for many physiological functions. Many vertebrates require ultraviolet (UV) radiation to perform different physiological processes. Artificial light is used to supplement UV in captive animals, through appropriate photoperiods and UV wavelengths. Previous studies reported that repeated exposure to artificial UV radiation may cause damage to the immune system. Taking into account the importance of UV effects and the serum complement system, the relationship between them was investigated. The study lasted 90 days and was carried out in plastic chambers. Ninety six broad-snouted caiman (*C. latirostris*) were assigned to four treatment groups with two replicates each: total darkness (TD), 8 hr per day (8 hr) and 16 hr per day (16 hr) of artificial UV/visible light exposure, and normal photoperiod of natural light (NP). Snout–vent length was measured to determine animal growth. Hemolytic assays were performed to evaluate the effects of artificial UV/visible light, TD, and NP on the serum complement

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system. Results showed that animals grew more in the NP group. The capacity of *C. latirostris* serum to hemolyze sheep red blood cells was higher in the NP group than when they are maintained in constant light–dark cycles (8 and 16 hr) or in TD. These data demonstrate that artificial UV should be considered as a potential hazard for captive crocodilians if it is not properly managed, and this should be taken into account in the general design of facilities for reptilian husbandry. Zoo Biol 31:523–533, 2012.

Keywords: immunity; complement system; crocodilian husbandry; raising; UV radiations

INTRODUCTION

Sunlight is a key environmental factor in almost all ecosystems, and the wide ecological effects of visible and infrared wavelengths have long been known. Basking by ectothermic vertebrates is thought to have evolved for thermoregulation [Allen et al., 1994; Johnson et al., 2008]. However, another beneficial effect of sunlight exposure, specifically the UVB component, includes endogenous production of vitamin D.

The spectral irradiance of ultraviolet (UV) radiation at the earth's surface is modified by temporal, geographical, and meteorological factors. Environmental photobiologists normally define the wavelength of UV regions as: UVC (200–280 nm), UVB (280–315 nm), and UVA (315–400 nm), with small variations in the specific definitions of these wavelength ranges [Frederick et al., 1989].

We can find increasing evidence that UV affects many trophic interactions and, in turn, influences a variety of ecosystem functions. Broad UV effects are classified into direct and indirect, based on the possibility that UV exposure induces modifications by affecting mechanisms of the organisms directly, or by producing alterations in some structure in the organism that, in turn, produces the final effect.

Reptilian husbandry is important to hobbyists and zoos, where many species are raised as components of conservation projects, as well as in farming ventures that raise them for pets, food, and raw materials. Reptiles are also used as laboratory models in bioscience research [Brames, 2007]. In conscientious husbandry programs, keepers must replicate the UV spectrum required for the photochemical process involved in the synthesis of vitamin D. UVB radiation is involved directly in vitamin D synthesis, which acts to regulate calcium and phosphorus absorption, both implied directly in the metabolism related to bone growth and maintenance [Lian et al., 1999]. If the correct UV range is not provided, calcium absorption will not be optimal [Brames, 2007] no matter what kind of diet, temperature, etc., is supplied.

Many reptile keepers and managers supply UV light to animals in order to offer or complement UV radiation (UVR) with the aim to mimic sunlight in the wild. Reptilian husbandry lamps contain three kinds of light spectrum: visible, UVA, and UVB. With respect to the visible light, it has been demonstrated to cause no suppression of the immune response [Kubasova et al., 1995], but instead, may directly modulate immune function; on the contrary, UVR has been shown to produce negative effects on the immune system (IS) [Roberts, 1995].

Likewise, Liu et al. [2006] demonstrated the importance of vitamin D in the human innate immune response, as insufficient vitamin D levels make it more difficult for cells to activate toll-like receptors, a type of receptor that mediates direct

immune response to bacterial threats. Also, there is considerable evidence that UVR, particularly in the UVB range, has pronounced effects on many normal functions of the IS in animals and human beings [Ilyas, 1986; Jeevan and Kripke, 1993; Schwarz, 2005]. Ilyas [1986] and Jeevan and Kripke [1993] emphasize that damage to the IS due to UVB could have far-reaching effects for the health of populations. The immunosuppressive properties of UVR are of major biological and clinical relevance [Schwarz, 2005]. More research has been focused on the effects of UV irradiation on cellular IS than on humoral IS [Hersey et al., 1983]. However, Artyukhov et al. [2007] established that UV light can modulate the activity of the serum complement system.

The serum complement system has been long appreciated as a major effector arm of the innate immune response. It consists of a complex group of proteins, which play an important role in host defense against infection, and provides an interface between the innate and adaptive immune responses. Soluble plasma proteins interact with one another in two distinct enzymatic activation cascades (the classical and alternative pathways) and in the nonenzymatic assembly of a cytolytic complex (the membrane attack complex) [Morgan, 2008]. A third pathway, the lectin pathway, which probably is as ancient as the alternative pathway, was discovered more recently [Matsushita and Fujita, 1995].

The serum complement system appears to be highly conserved in vertebrates, although research in reptilian and amphibian species is scarce. In some ectothermic species, the serum complement system is present in multiple forms, and researchers have hypothesized that this serum complement diversity has been used as an evolutionary strategy to expand innate immune recognition capabilities [Sunyer et al., 1998]. The serum complement system has been identified in some species of crocodilians [Merchant et al., 2005; Merchant and Britton, 2006] including *C. latirostris* [Siroski et al., 2010], and various antimicrobial activities of crocodilian tissue have been described [Shaharabany et al., 1999; Merchant et al., 2003; Siroski et al., 2009]. This effective innate immune component is suspected as a very important factor for the resistance of microorganism attack, being possibly one of the reasons for crocodilian longevity [Siroski et al., 2010].

Many studies were conducted on UV effects in humans [Marrot and Meunier, 2008] and marine invertebrates [Hader et al., 2007; Pruski et al., 2009]. However, few efforts were made to investigate the effects of UV exposure on wildlife. Despite the fact that serum complement system is a relevant component of innate immune response in crocodilians, UV effects on it has not yet been investigated. The goal of this study was to evaluate the effect of artificial UV/visible light exposure on *Caiman latirostris* growth and serum complement system.

MATERIALS AND METHODS

The study lasted for 90 days. It was carried out in eight plastic chambers (96 cm long, 41 cm wide, and 42 cm high, surface = 0.39 m^2). They were tilted to give 60% dry and 40% water surface areas, with a maximum water depth of approximately 15 cm. Inside the chambers, temperatures were maintained at $31 \pm 2^{\circ}C$ and were monitored with Hobo Temperature recorders (Onset Computer Corp., Pocasset, MA) at all treatments. Experimental treatments were divided into: total darkness (TD), 8 hr per day (8 hr) and 16 hr per day (16 hr) of artificial UV/visible light exposure, and normal photoperiod of natural light (NP) which was 9.5–10.5 hr light

during the study, approximately. The 8- and 16-hr UV/visible light treatments were under TD when the light was out, during all the study. Artificial UV/visible light was provided by an 18-W visible-UV light lamp used in reptilian husbandry (UVA 30% and UVB 5%; Sylvania Reptistar®, Capital Federal, Argentina) which was set at a height of 0.45 m from the base of the plastic chambers and provided a total irradiance of 45.73 W/m^2 (Irradiance = Lamp power/plastic chamber surface, including both visible and UV components). The chambers of all treatments were covered with shade cloth (with 95% of solar irradiation filter) up to the half of both replicates, including part of dry and water surface areas, in order to provide a shaded area to the animals. The irradiance received under shaded surface in the UV-treated groups was 2.29 W/m^2 (95% of UV/visible radiation was filtered). In the case of NP treatment, the chambers were covered also by low-density nylon (Agrotileno IPESA®, Ciudadela, Buenos Aires, Argentina) with 2% UV filter. The study was carried out in Santa Fe province, Argentina, where solar irradiance average during the time of the year when the study was conducted is 150 W/m^2, approximately [Ceballos et al., 2005]. Based on the conditions previously described, each chamber at NP treatment was exposed to an irradiance of 147 W/m^2 (150 W/m^2—the 2% filtered by nylon) and the portion of the chamber under the shade cloth was exposed to 4.5 W/m^2 (150 W/m^2—the 97% filtered together by nylon and shade cloth). It is important to note that this irradiance corresponds to the total range of solar light that reach the earth surface, without distinction between different light spectrums.

Ninety six broad-snouted caiman (*C. latirostris*) approximately 5 months, hatched from four nests harvested in the wild in the same season and artificially incubated as part of Proyecto Yacaré (PY) ranching program [Larriera et al., 2008], were selected for the study. They were similar in size and apparently healthy. All the caimans used in the experiment had been maintained in pools at PY commercial husbandry facilities since hatching. During the experiment, they were maintained under identical conditions (temperature, food composition, feed and cleaning regimes, etc.) [Larriera and Imhof, 2006]. Each animal was individually marked with foot webbing tags (Monel Natl Band and Tag CO., Newport, Kentucky, 1005-1), and three animals from each nest were randomly assigned to each of two replicates (12 caimans per replicate) of the different treatment groups. All animals were treated following the Reference Ethical Framework for Biomedics Researches: ethical principles for research with laboratory, farm, and wild animals [National Scientific and Technical Research Council, 2005], using techniques of blood collection and minimizing stress and suffering by suitable management methods.

Growth was evaluated by snout–vent length (SVL) measured at the beginning and at the end of the study (precision = 0.1 cm). At the end of the experiment, blood was collected from each animal via the spinal vein [Zippel et al., 2003] using a 25-ga needle and a 5-ml syringe. About 1 ml of blood was transferred to a serum Vacutainer™ (Franklin Lakes, New Jersey) tube. The blood was allowed to clot at room temperature, and serum was removed after centrifugation at 2,500 *g* for 15 min (room temperature).

To evaluate the effects of different time periods of UV/visible light exposure on the *C. latirostris* serum complement system, we used the sheep red blood cell (SRBC) hemolysis assay. This assay is based on the hemolytic disruption of SRBCs by means of immunological proteins of the serum [Mayer, 1967]. It was adapted to crocodilian serum by Merchant et al. [2006] and has been applied to characterize the

C. latirostris serum complement system [Siroski et al., 2010]. Fresh SRBCs were obtained from heparinized whole blood collected from Merino sheep (*Ovis aries*) in Estación Zoológica Experimental (Santa Fe Province, Argentina). Sheep blood was washed with phosphate-buffered saline (pH 7.4) four times until supernatant was clear, and then a 2% SRBCs (v/v) solution was prepared.

The hemolytic assay was performed to detect the serum complement system activity of broad-snouted caiman serum, including two classical inactivators of serum complement, mild heat treatment and ethylenediaminetetraacetic acid (EDTA). Untreated caiman serum, preheated serum (56 °C for 30 min), and serum treated with 50 mM EDTA were exposed to 1% (v/v) SRBCs. Serum samples included in this determination consisted of a pool from the animals exposed to a normal photoperiod. Another assay was performed to evaluate UV exposure effects on broad-snouted caiman serum complement system. For this study, 0.5 ml of serum from each caiman of each treatment was combined with 0.5 ml of 2% SRBC (v/v) and incubated at room temperature for 30 min. After incubation, the mixture was centrifuged at 2,500 *g* for 5 min at room temperature. Then 300 µl of the supernatant was transferred to a microtiter plate and the optical density was measured at 540 nm in a microplate reader (Labsystem Multiskan RC, Helsinki, Finland).

With the aim to obtain a positive control, a solution of 1% SRBCs containing 0.1% (v/v) Triton X-100 was aggressively absorbed and ejected several times through a tuberculin syringe until complete hemolysis was achieved. Full hemolysis was confirmed by means of an optical microscope (Olympus BH-2; Olympus Co., Japan) at 400 × magnification. Positive control aliquots were centrifuged, and the optical density of the supernatant was measured in a microplate reader at 540 nm (considered as the 100% hemolysis). All experiments were conducted in triplicate to obtain valid statistical evaluation of the results. The results are expressed as percentage of maximum hemolysis (%MH; mean ± standard error).

Statistical analysis was performed using SPSS 16.0 software [SPSS for Windows, 2007]. Data were tested for normality with Kolmogorov–Smirnov test, and homogeneity of variances between groups was verified by Levene test. One-way ANOVA was used to test differences in growth (final SVL−initial SVL) and %MH from animals exposed to artificial visible/UV light, TD, and NP. Same analysis was applied to determine differences in %MH of untreated broad-snouted caiman serum, preheated serum, and serum treated with EDTA. Significant differences among groups were detected by Tukey's Test, and a P value of ≤ 0.05 was considered statistically significant.

RESULTS

Caimans exposed to NP grew more (13.22 ± 2.52 cm) than all other groups ($P < 0.001$), but no differences were observed between 8 hr (6.13 ± 2.88 cm), 16 hr (8.91 ± 2.19 cm), and TD (7.44 ± 1.98 cm) groups. There was a clear clutch effect in SVL ($P < 0.001$; Fig. 1).

Assays with unsensitized SRBCs were conducted to characterize *C. latirostris* serum complement system. The data depicted in Figure 2 demonstrate the ability of untreated broad–snouted caiman serum (91 ± 7.1%) to rupture SRBC membranes compared with serum treated with serum complement system classical inactivators: EDTA (13 ± 6.2%) and heat treatment (17 ± 5.2%). The absorbance recorded

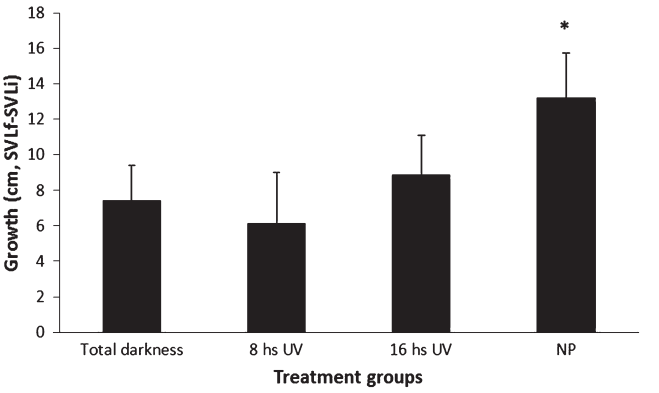

Fig. 1. SVL values of caimans maintained during 90 days under TD, 8 and 16 hr per day UV/visible light, and natural photoperiod (NP). *Statistically significant compared with the other groups ($P<0.001$; ANOVA–Tukey test). SVL, snout–vent length; UV, ultraviolet; TD, total darkness.

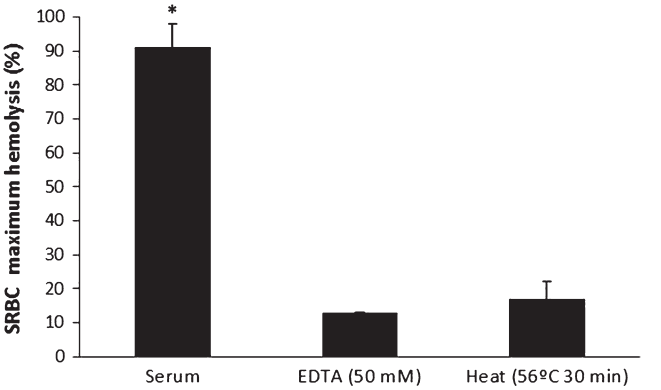

Fig. 2. Effects of treated and untreated serum on the hemolysis of SRBCs by broad-snouted caiman serum. Untreated caiman serum demonstrated higher ability to disrupt SRBCs compared with treated serum with two classical inhibitors of complement system: EDTA (50 mM) and Heat (56°C 30 min). *Statistically significant compared with the other groups ($P<0.001$; ANOVA–Tukey test). SRBC, sheep red blood cell.

indicated that there were significant reductions in SRBC maximum hemolysis when serum complement system inhibitors were added ($P<0.001$).

Differences were not detected in serum-mediated SRBC hemolysis between caimans from different clutches, nor between individuals assigned to the various treatment groups ($P>0.05$). Figure 3 refers to the results of SRBC hemolysis of serum from broad-snouted caiman exposed to TD, artificial UV/visible, and NP. The effects of both artificial UVR regimes, as well as TD, affect the ability of caiman serum to disrupt the integrity of SRBCs ($P<0.001$). The lowest activity of caiman serum was identified in the group of animals exposed to UV light for 16 hr per day ($31.11 \pm 7.9\%$) followed by the group exposed for 8 hr per day ($36.24 \pm 5.28\%$) and

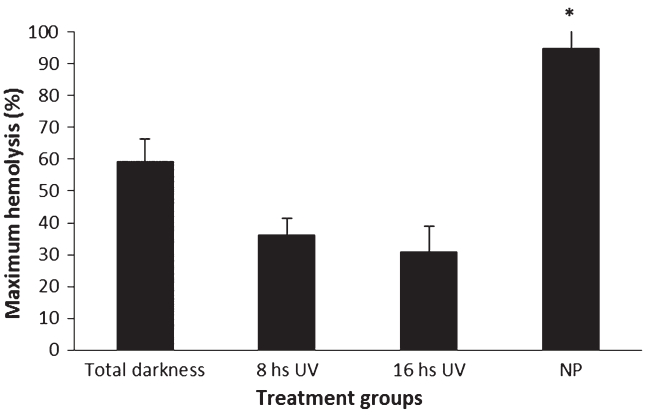

Fig. 3. Broad-snouted caiman serum showing different hemolysis abilities between groups maintained during 90 days under TD, 8 and 16 hr per day UV/visible light, and NP. Results are presented as mean percentage of maximum hemolysis (MH%) for three determinations. *Statistically significant compared with the other groups ($P<0.001$; ANOVA–Tukey test). NP, natural photoperiod; UV, ultraviolet; TD, total darkness.

then the group in TD ($59.2\pm7.31\%$). The group maintained under natural photoperiod showed the highest hemolysis ability ($94.8\pm6.9\%$).

DISCUSSION

Basking by ectothermic vertebrates is thought to have evolved for thermoregulation, and other beneficial effects of sunlight exposure, such as fever response to infection [Vaughn et al., 1974; Burns et al., 1996; Merchant et al., 2007], removal of external parasites and commensals [Hutchinson, 1989], and stimulation of vitamin D3 synthesis [Ferguson et al., 2003]. Based on these examples, it is not surprising that there are many positive effects of sunlight on human and animal health that facilitate fundamental biological functions, such as growth and IS [Roberts, 2005].

Some studies on reptiles have showed that sunlight is more convenient than artificial light to obtain a better growth of animals under captivity. Sunlight provides the suitable quantity and quality of UVR for organism to develop vital process [Ferguson et al., 2005; Karsten et al., 2009]. The better growth we evidenced under NP is consistent with this statement.

In contrast with adaptative immunity, which is restricted to vertebrate animals, innate immunity is more ancient and is used by invertebrates such as insects and echinoderms, as well as by vertebrates [Song et al., 2000]. Recent studies in crocodilians have shown that they have a strong serum complement system activity, and that it could be a very important innate immune component which contributes to their high resistance to attack by microorganisms [Merchant et al., 2005; Merchant and Britton, 2006; Siroski et al., 2010].

Gallo et al. [1989] reported that UVR has an important effect on the IS. Several studies have shown that the serum complement component C3 may be involved with the induction of UV immunosuppression and antigenic tolerance [Hammerberg

et al., 1998]. Livden et al. [1987] showed that serum levels of healthy individuals' serum complement components C3 and C4 were not affected after 10, 20, or 28 days of UVA exposure, but C4 was significantly reduced and C3 was significantly increased by UVB irradiation. Activation of C3 by UVB was indicated by the detection of serum complement deposits of C3b and C3d in epidermal cells within 24 hr of exposure [Rauterberg et al., 1993]. UVB exerts enhancing effects on the production of serum complement component C3 by interferon-γ-stimulated cultured human epidermal keratinocytes, in contrast with photochemotherapy and UVA radiation that show suppressive effects [Terui et al., 2001]. In this study, we have found that serum complement system activities measured by SRBC hemolysis assays under natural photoperiods were of similar percentage to that shown in previous studies in our lab [Siroski et al., 2010]. The data revealed that hemolytic activity decreased when caimans were exposed to different time periods of artificial UVR and TD (Fig. 3). The lowest activity was measured in serum from animals exposed to longer periods of UVR (16 hr) and activity increased when exposure time decreased (8 hr and total darkness). Through the use of specific serum complement inhibitors, we can affirm hemolytic activities are due to serum complement system [Siroski et al., 2010].

In absolute values, total irradiance received between groups is significantly different. The caimans exposed to NP received more total UV/visible irradiance ($147\,W/m^2$) than other treatments. All the animals had the possibility to regulate their exposure by limiting it under shade cloth, as it was evidenced in panther chameleons (*Furcifer pardalis*), when they behaviorally adjusted their exposure to UV to regulate their internal vitamin D3 status [Karsten et al., 2009]. Therefore, they could expose themselves to full or partial sun during midday, when UV irradiance is highest [Lu et al., 1992].

We suspect that the reduction in serum complement system activity at 8 and 16 hr per day of artificial UV/visible light demonstrates a UVA-B dose-dependent response, which might affect the IS of captive herpetofauna. Similar reports were obtained in mice using experimental skin lesions, in which long UVR is the major factor responsible for this pathology [Natali et al., 2005]. Up to now, the details of how UV exposure may suppress the immune responses have not been elucidated.

Previous studies adjudicated the negative effects observed on IS to UVA, UVB, or both. Influences of UVB on immune parameters modulation, and the fact that UVA radiation has only minor effects has been shown previously [Jokinen et al., 2000]. In this study, we could identify only the significant functional disorder of serum complement system activity caused by artificial UVR, but based on the UV lamp characteristics, we cannot accurately determine if it was due to the effects of UVA, UVB, or both.

The benefit of artificial light as a UV supplement for captive herpetofauna depends on appropriate photoperiod and UV wavelength exposure [Caliman Filadelfi et al., 2005]. However, our results clearly show the importance of offering areas with natural light and shade simultaneously. A regulatory mechanism for exposure probably exists in these animals similar to that detected in chameleon for vitamin D3 synthesis [Ferguson et al., 2002]. We provided light and shade areas to offer the animals the chance to regulate their own exposure. As we did not have any information about how much time the caimans spent in the shade, we cannot make any assertion about the specific role shade played, but we think it

could have a potential contribution in regulation that should be clarified in further studies.

UVR can cause innate immune activity alteration, which could possibly lead to the impaired response of exposed individuals to infections, increasing their incidence and making them more lethal [Leaf, 1993].

Our results demonstrated that artificial UV should be considered a potential hazard if it is not properly managed and this should be taken into account by those dedicated to reptilian husbandry and maintenance facilities design. Sunlight exposure at normal photoperiod seems to be the better alternative for providing proper amount and quality of UVR to caimans in captivity. Anyway, if artificial UV light is required, it should be combined with shaded area in order they can self-regulate exposure.

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