

## **Detection and Characterization of Phospholipase A2 (PLA2) in Caiman latirostris and Caiman yacare Plasma**

Authors: Siroski, Pablo A., Merchant, Mark E., Poletta, Gisela L., Larriera, Alejandro, and Ortega, Hugo H.

Source: Zoological Science, 30(1) : 35-41

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.30.35>

---

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at [www.bioone.org/terms-of-use](http://www.bioone.org/terms-of-use).

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

---

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

# Detection and Characterization of Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in *Caiman latirostris* and *Caiman yacare* Plasma

Pablo A. Siroski<sup>1,4\*</sup>, Mark E. Merchant<sup>2</sup>, Gisela L. Poletta<sup>3</sup>,  
Alejandro Larriera<sup>1</sup>, and Hugo H. Ortega<sup>4</sup>

<sup>1</sup>Proyecto Yacaré - Laboratorio de Zoología Aplicada: Anexo Vertebrados  
(FHUC - UNL / MASPyMA), (3000) Santa Fe, Argentina – CONICET

<sup>2</sup>Department of Chemistry, McNeese State University  
(Box 90455) Lake Charles, LA 70609, USA

<sup>3</sup>Cátedra de Toxicología y Bioquímica Legal, Facultad de Bioquímica y Ciencias Biológicas,  
Universidad Nacional del Litoral (3000) Santa Fe; FCEN - UBA, Bs. As., Argentina – CONICET

<sup>4</sup>Departamento de Ciencias Morfológicas, Facultad de Ciencias Veterinarias,  
Universidad Nacional del Litoral (3000) Santa Fe, Argentina – CONICET

---

Reptiles have proven to have a versatile and efficient nonspecific immune system adapted to the environments in which they commonly live. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is important hydrolytic enzyme involved in the regulation of specific types of messengers, with significant roles in the innate immune response. A number of agents that exert effects on cellular receptors emit a series of signals leading to the increased activity of PLA<sub>2</sub>. Phospholipase A<sub>2</sub> has been identified and characterized in temperature, plasma concentration, and kinetic dependence in two species of caiman. The results of these studies suggest that the high PLA<sub>2</sub> activities observed in caiman plasma may be an important component of a well-developed innate immunity. Based on the knowledge of their properties, this powerful immunologic component should be evaluated as a possible application in the veterinary or even human therapeutic industry. Additionally, this is another reason to consider these animals excellent models for the study of immune phylogenetic mechanisms.

**Key words:** phospholipase, PLA<sub>2</sub>, immune system, crocodylians, innate immunity

---

## INTRODUCTION

Reptiles have exhibited a versatile and efficient nonspecific immune system adapted to the environments in which they commonly live. Crocodylians display strongly marked social behaviors and responses to stressors that can trigger serious disputes between co-specifics, predators, and even conflict with human activities (Webb and Messel, 1977; Webb and Manolis, 1983). As a result, they may show trauma, serious injuries and sometimes the loss of entire limbs. Generally, these animals live in environments (both natural and captive) that contain high concentrations of pathogenic microorganisms. In many cases, the combination of these factors could cause a local or systemic infection. However, crocodylians tolerate these situations and generally do not show signs of illness (Siroski et al., 2009).

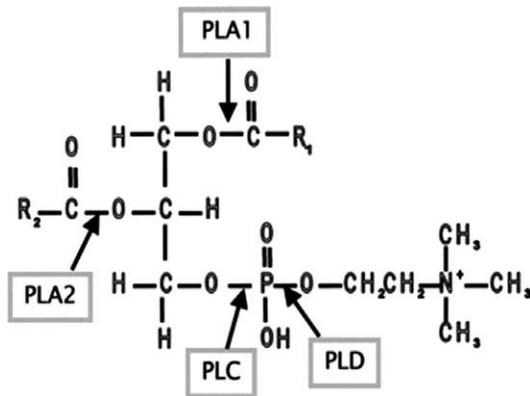
The cells of the innate immune system recognize pathogens by means of a series of extracellular and intracellular receptors, such as the Fc receptor, mannose receptor, and Toll-like receptor (TLR). The recognition of pathogens stimulates the synthesis of inflammation mediators called eicosanoids. These mediators are derived from arachidonic acid

(AA), a polyunsaturated omega-6 fatty acid that organisms can incorporate directly through the diet, or synthesize from linoleic acid (Balsinde and Winstead, 2002). The AA never circulates in the free acid form, but is esterified to the sn-position of membrane glycerophospholipids. Therefore, prior to the synthesis of eicosanoids, the AA has to be released from phospholipids. The enzymes involved in that release are phospholipases A<sub>2</sub> (PLA<sub>2</sub>) (Fig. 1) (Astudillo et al., 2009). These enzymes catalyze the hydrolysis of glycerophospholipids, generating free fatty acid and a lysophospholipid, which serves as the main source of AA released during cell stimulation (Kudo and Murakami, 2002).

The free AA is the precursor of eicosanoids, which include prostaglandins (generated through reactions catalyzed by cyclooxygenase), leukotrienes, and lipoxins (generated through reactions catalyzed by lipoxygenase) (Funk, 2001). When lysophospholipid has a choline group as a head group and an alkyl group in sn-1 position, the PLA<sub>2</sub> reaction generates a precursor of platelet activating factor (PAF). Therefore, PLA<sub>2</sub>s are important enzymes involved in signaling, as they regulate the generation of specific types of secondary lipids messengers with significant roles in innate immune response. Although messenger pathways that mediate the production of eicosanoids by cells involved in innate immunity are not well characterized, all of these groups have been implicated in various types of lipid metabolism and in the progression of several diseases. Given the

\* Corresponding author. Tel. : +54-342-4579256;  
Fax : +54-342-4579256;  
E-mail : psiroski@fcv.unl.edu.ar

doi:10.2108/zsj.30.35



**Fig. 1.** Schematic diagram of a phospholipid (i.e. phosphatidylcholine) hydrolysis caused by various phospholipases. PLA<sub>1</sub>, phospholipase A<sub>1</sub>; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLC, phospholipase C; PLD, phospholipase D; R<sub>1</sub>/R<sub>2</sub>, variable fatty acid residues (Budnik and Mukhopadhyay, 2002).

clinical importance of PLA<sub>2</sub> enzymes, they are of interest for pharmaceutical industry and biotechnology, regarding the development of selective and potent inhibitors of each of these phospholipases (Burke and Denis, 2009).

In general, based on their structures and enzymatic properties, phospholipases are focused in regulatory functions of each isoenzyme (Murakami et al., 1997). A number of agents that exert effects on cellular receptors of the innate immune system emit a series of signals leading to an increased activity of PLA<sub>2</sub>. These signaling pathways have been studied over the past 20 years (Astudillo et al., 2009).

The PLA<sub>2</sub> enzymes are widely distributed in animal tissues and body fluids, and play an important role in inflammation and inflammatory diseases (Nevalainen et al., 2000; Six and Dennis, 2000), as well as in the effectiveness of killing bacteria *in vitro* (Koduri et al., 2002) and *in vivo* (Laine et al., 1999). The bactericidal effect is based on the hydrolysis of the phospholipid component of bacterial cell membrane by PLA<sub>2</sub> (Nevalainen et al., 2009), which perturbs membrane trafficking and modulates intracellular bacterial growth (Mansueto et al., 2012). PLA<sub>2</sub> was found to be the major antibacterial factor in human acute phase serum against the gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*, exceeding complement in efficiency (Perrin-Cocon et al., 2004). Among other functions, it can induce NF- $\kappa$ B activation in macrophages, as well as elastase release in polymorphonuclear leukocytes (Silliman and Moore, 2002). In addition, its important participation in the immune system was tested by inhibiting activity generated for the entomopathogenic bacterium *Xenorhabdus nematophila*, which induced immunosuppression (Shrestha and Kim, 2009).

Phospholipase A<sub>2</sub> enzymes are toxic components detected and characterized in the venoms of some reptiles, including most venomous snakes (Kini, 2003), *Heloderma* lizards (Vandermeers et al., 1991) and members of the Varanidae family (Fry et al., 2006). Recently, PLA<sub>2</sub> has also been described in crocodylian plasma. Merchant et al. (2009) demonstrated its presence in *Alligator mississippiensis*, and Nevalainen et al. (2009) in *Crocodylus siamensis*, *C.*

*porosus* and *C. siamensis* hybrids with *C. porosus*. These enzymes have also been reported in the plasma of *C. niloticus*, *Mecistops cataphractus*, and *Osteolaemus tetraspis* (Merchant et al., 2011). To our knowledge, PLA<sub>2</sub> plasma activity in Caiman genus has not yet been studied. Due to the important role of PLA<sub>2</sub> enzymes in the generation of the innate immune messengers, the aim of this study was to identify and characterize the activity of this enzyme in *C. latirostris* and *C. yacare* plasma.

## MATERIALS AND METHODS

### Animals

For this study, samples were collected from wild *C. latirostris* in the Province of Santa Fe, San Cristóbal Department ("El Fisco" Natural Reserve; S 30°11'26", W 61°0'27") and wild *C. yacare* from the Province of Corrientes (Ibera Wetland, Tabe Lagoon; S 27°38'55.87", W 56°32'0.26") in Argentina. These animals were captured in public and private wetlands that have large populations of these species. These areas were selected because they have experienced little modification by human activities, to avoid any bias in the results.

Samples were obtained from adult wild *C. latirostris* ( $n = 13$ ; seven females and six males, from 1.51 to 2.31 m of total length) and *C. yacare* ( $n = 14$ ; seven males and seven females from 1.44 to 2.11 m of total length). It should be noted that, due to the influence of temperature on the physiology of these animals in both cases, the samples were collected during the summer with a few days between captures of one species and another. Animals were measured and returned to their environment within an hour of capture. The blood samples were collected relatively quickly after capture to avoid an increase in corticosterone concentration (a hormone directly related to stress in crocodylians), which could affect the results (Lance and Lauren, 1984; Turton et al., 1997).

Blood samples were obtained from spinal vein (Zippel et al., 2003) using heparin as an anticoagulant. Whole blood was centrifuged immediately at 4500 *g* for 20 minutes, at room temperature (approximately 24°C). The plasma was frozen at -20°C and PLA<sub>2</sub> enzyme assays were conducted within seven days of capture.

### Bacterial culture

For PLA<sub>2</sub> assays, *Escherichia coli* ATCC 11105 strain was used to inoculate 1 L of nutrient broth. Subsequently, 1 mg of BODIPY® FL C16 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid; Invitrogen, Carlsbad, CA, USA) dissolved in 1 ml of DMSO (dimethyl sulfoxide) was added to this culture and incubated at 37°C for 18 h. The culture was then centrifuged at 8000 *g* for 15 min. The supernatant was discarded and the bacterial pellet resuspended in a solution of 0.9% NaCl (*w/v*). This process was carried out twice. Finally, the pellet was resuspended in 30 mL assay buffer (1 mM Ca<sup>2+</sup> in 100 mM tris-HCl, pH 7.4).

### PLA<sub>2</sub> determination assay

This assay was developed to measure the secretory PLA<sub>2</sub> enzyme activity in plasma. We used fluorescent BODIPY® to label a fatty acid that binds occupies the *sn*-2 position of phospholipids. Bacterial cultures add these macromolecules as component of the outer membrane during growth and proliferation. When bacteria are exposed to the activity of the PLA<sub>2</sub> enzyme, the labeled *sn*-2 fatty acid is hydrolyzed from the membrane and released into the assay buffer. After a brief centrifugation, the pellet formed contains bacteria with the labeled fatty acid in the membrane, while the supernatant has free labeled fatty acid that can be measured by spectrofluorometry. PLA<sub>2</sub> activity was measured in a spectrofluorometer at an excitation  $\lambda$  of 500 nm and an emission  $\lambda$  of 510 nm, and excitation and emission slit widths of 1 nm.

### Caiman PLA<sub>2</sub> activity temperature dependence assay

To evaluate the effect of temperature on enzyme activity, PLA<sub>2</sub> assays were performed at different temperatures (from 5°C to 40°C, with intervals of 5°C). Prior to the onset of the reaction, aliquots of start buffer (950 µL), labeled bacteria solution (50 µL) and caiman plasma (100 µL) were incubated separately for 15 min to the corresponding temperature. Components of the reaction were next mixed and incubated for 30 min at the same temperatures, after which 900 µL of stop buffer (50 mM EGTA in 100 mM Tris-HCl, pH 8.0) were added. The mixture was centrifuged at 16,000 *g* for 5 min and the product in the supernatant was measured by spectrofluorometry.

### Caiman PLA<sub>2</sub> activity plasma concentration dependence assay

To determine the effect of plasma concentration on PLA<sub>2</sub> activity, different amounts of caiman plasma (0, 1, 2, 5, 10, 20, 50 and 100 µL) were added and then balanced to a final volume of 750 µL with start buffer. Then, 100 µL of the solution containing labeled *E. coli* were added and incubated for 30 min at room temperature (25°C). After incubation time, the reaction was stopped with the addition of 750 µL stop buffer, then centrifuged, and the fluorescence intensity of supernatant determined by spectrofluorometry.

### Caiman PLA<sub>2</sub> activity time dependence assay

To evaluate the kinetics of the PLA<sub>2</sub> enzyme activity 3.5 ml of plasma, 1.75 mL of the solution containing the fluorescent marked bacteria and 33.25 mL of start buffer were mixed at room temperature (25°C). At different time intervals (0, 5, 10, 15, 20, 30, 60, 90 min), 1.1 mL of the mixture was removed and added to 900 µL of stop buffer at the same temperature, centrifuged, and the supernatant was measured by spectrofluorometry, as described previously.

### Inhibition of PLA<sub>2</sub> enzyme activity

We conducted an assay in which plasma of both caiman species were exposed to p-Bromophenacyl bromide (BPB), considered a specific inhibitor of PLA<sub>2</sub>. Different concentrations of BPB (0, 1, 5 and 10 mM) were incubated with caiman plasma for 30 min at 25°C. The reaction was stopped as previously described, the sample centrifuged, and the fluorescence of the supernatant measured spectrofluorometrically.

### Statistical analysis

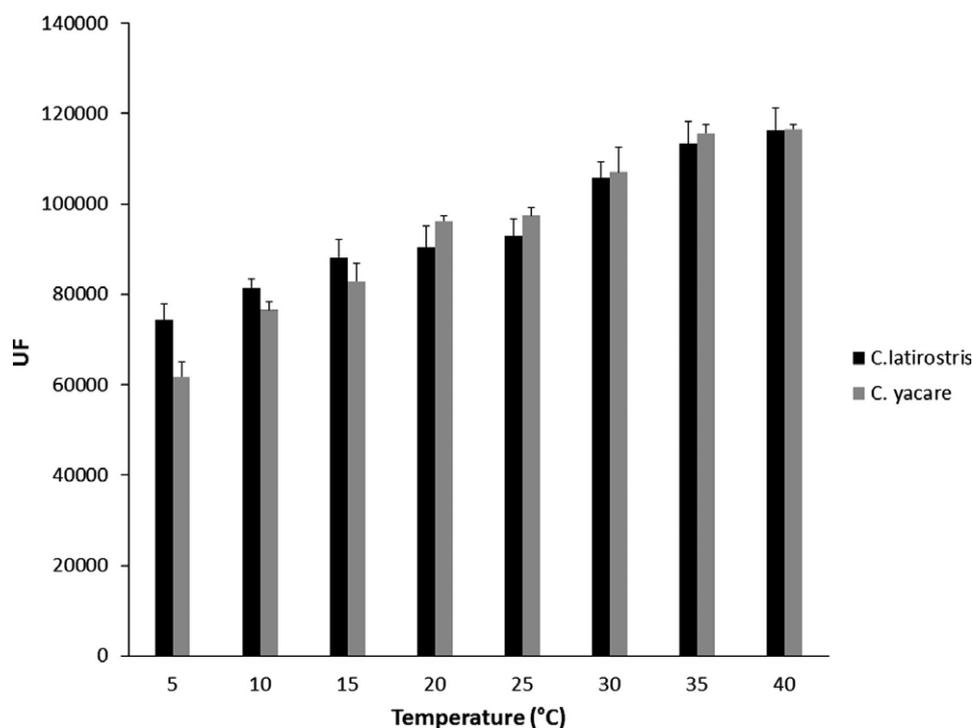
All assays were performed in quadruplicate and the results are expressed as fluorescence units (FU) ± standard error (SE). The data were tested in normality by the Kolmogorov-Smirnov test, and homogeneity of variances using the Levene test. Regressions with robust standard errors for repeated measures (Dawson and Trapp, 2001) were used to analyze the effect of temperature, kinetics, and concentration on PLA<sub>2</sub> activity for each species and to compare results between them. Differences in PLA<sub>2</sub> activity between sexes were analyzed by T-test, and the effect of BPB on PLA<sub>2</sub> activity by one-way ANOVA followed by Tukey's test. Values of  $P \leq 0.05$  were considered statistically significant.

## RESULTS

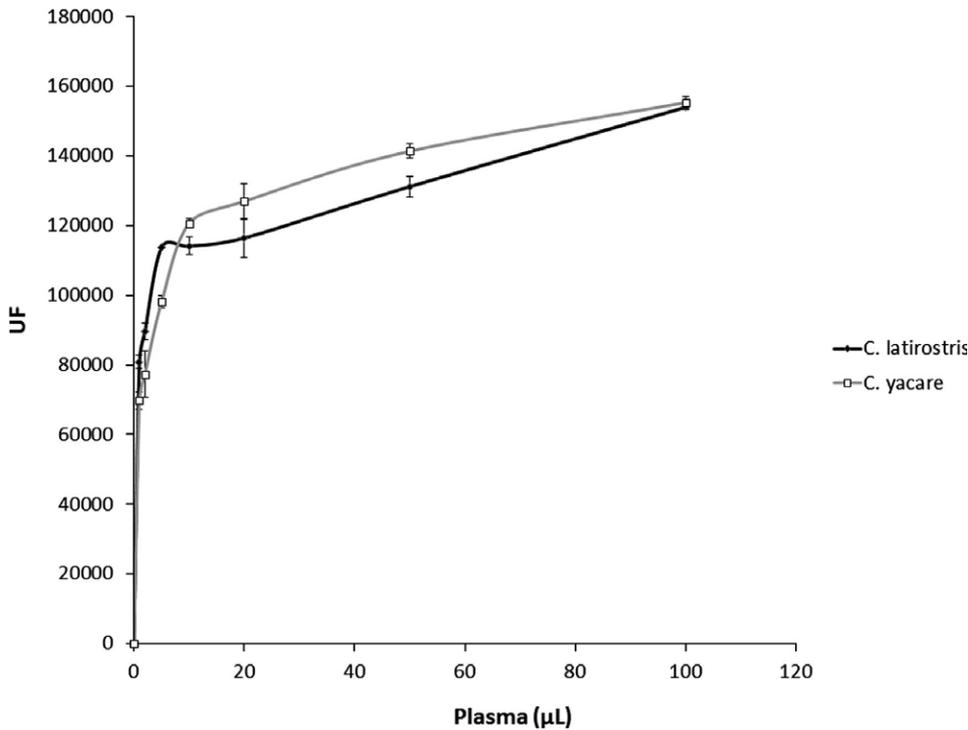
Plasma PLA<sub>2</sub> activity in both Caiman species demonstrated a positive relationship with temperature (Fig. 2). At the lower temperatures (5, 10 and 15°C), PLA<sub>2</sub> activity recorded was significantly higher ( $P < 0.05$ ) in *C. latirostris* (74,267.75 ± 3552.93 UF, 81,582 ± 1805.5 UF, and 88,225 ± 3953.5 UF, respectively) than in *C. yacare* (61,812.78 ± 3258.34 UF, 76,609 ± 1805.51 UF, and 82,875.25 ± 3953.55 UF, respectively); while at the rest of the temperatures studied, the activity recorded was slightly higher in *C. yacare* (20°C: 96,237.08 ± 1247.16 UF, 25°C: 97,487.43 ± 1863.9 UF, 30°C: 107,084.2 ± 5512.38 UF, 35°C: 115,675.8 ± 1991.25 UF, and 40°C: 116,606.3 ± 959.9 UF) than in *C. latirostris* (20°C: 90,349.5 ± 4669.7 UF, 25°C: 92,848.5 ± 3822.71 UF, 30°C: 105,709.8 ± 3588.37 UF, 35°C: 113,351 ± 4852.85, and 40°C: 116,305.3 ± 5050.85 UF), but not significantly so.

Phospholipase A<sub>2</sub> activity increased gradually at a rate of 1209.663 UF/°C for *C. yacare* ( $R^2 = 0.964$ ,  $P < 0.001$ ), and 1554.203 UF/°C for *C. latirostris* ( $R^2 = 0.968$ ,  $P < 0.001$ ). The highest activities were recorded at 40°C in both species.

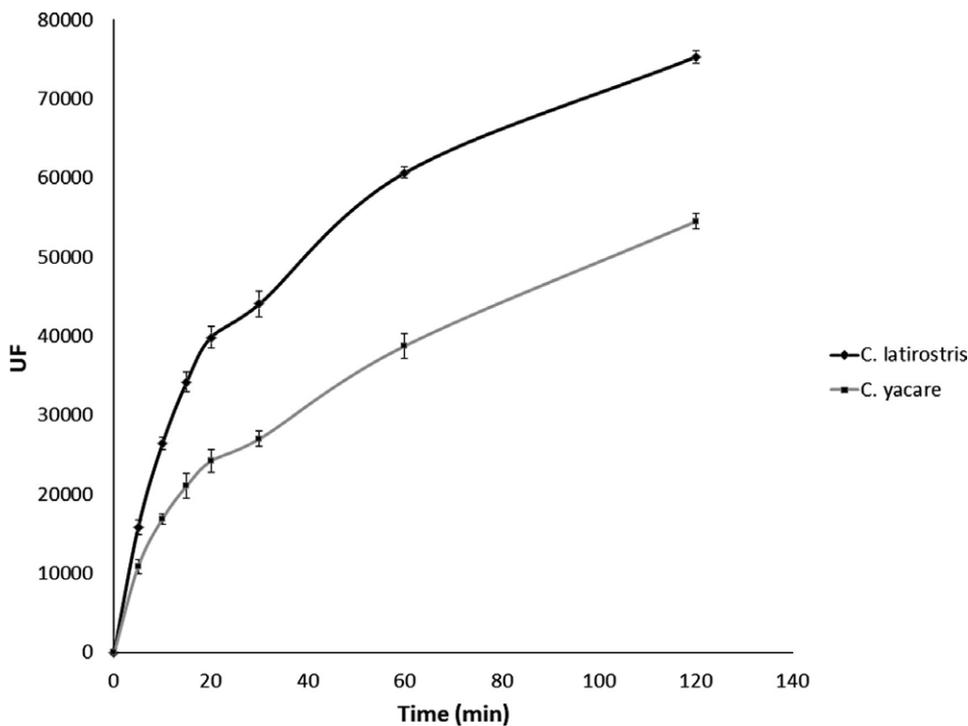
*C. latirostris* and *C. yacare* PLA<sub>2</sub> plasma activity showed a positive relationship with increasing plasma concentration (Fig. 3). Incubating 100 µL of bacteria labeled solution with 1, 2, 5, 10 µL of *C. latirostris* plasma displayed increase activity (80,824.68 ± 1849.83 UF; 89,639.50 ± 2420.90 UF; 113,669.50 ± 160.61 UF and 114,076.81 ± 2475 UF, respectively), more than *C. yacare* plasma (69,723.28 ± 2526.84 UF; 77,327.38 ± 6603.48 UF; 98,056.82 ± 1806.63



**Fig. 2.** Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activities in caiman plasma increased with incubation temperature (*C. yacare*:  $R^2 = 0.964$ ,  $P < 0.001$ ; *C. latirostris*:  $R^2 = 0.968$ ;  $P < 0.001$ ). Samples were analyzed in quadruplicates, and are presented as the means ± standard deviations.



**Fig. 3.** The effects of rising *C. latirostris* and *C. yacare* plasma concentration caused an increase in the reaction product by PLA<sub>2</sub> activity, which is reflected in the fluorescence intensity recorded (*C. latirostris*:  $R^2 = 0.531$ ,  $P < 0.05$ ; *C. yacare*:  $R^2 = 0.611$ ;  $P < 0.05$ ).



**Fig. 4.** PLA<sub>2</sub> activity in plasma of both species at different times showed similar behavior but with a greater activity in *C. latirostris* (*C. latirostris*:  $R^2 = 0.886$ ; *C. yacare*:  $R^2 = 0.804$ ,  $P < 0.01$ ).

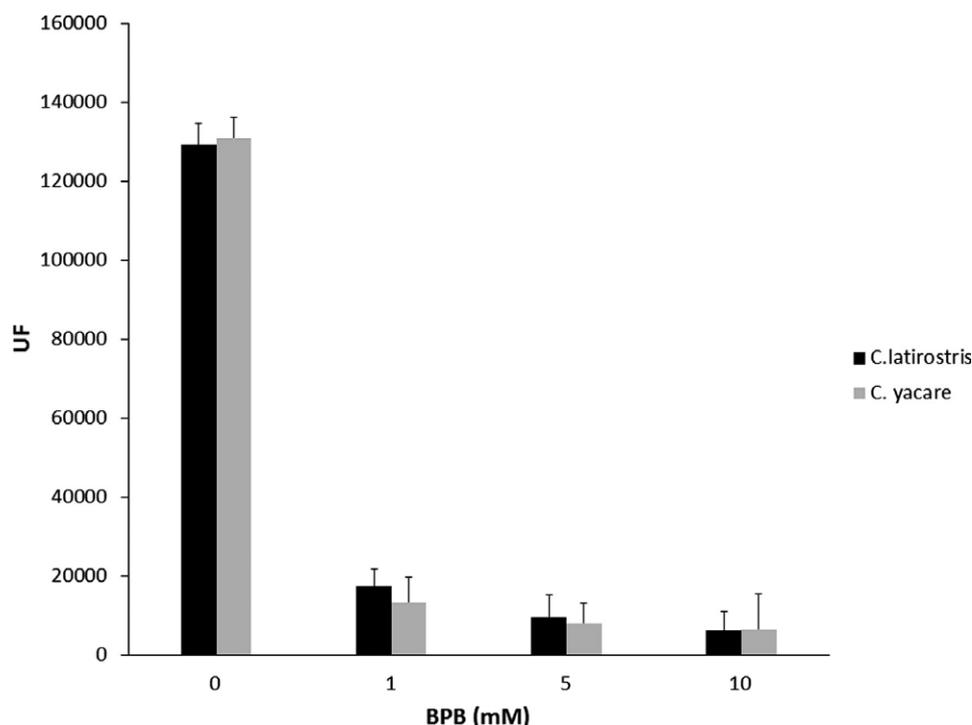
UF y 120,648 ± 1413.51 UF, respectively). From the addition of 20 µL, the ratio of PLA<sub>2</sub> activity was reversed, and nearly equalized in the last volume of plasma added *C.*

*latirostris*, 20 µL: 66,469.18 ± 5575.08 UF; 50 µL: 131,182.82 ± 3028.24 UF; 100 µL: 153,989.15 ± 606.75; and *C. yacare*, 61,977.89 ± 51,68.06 UF; 50 µL: 141,369.25 ± 2066 UF; 100 µL: 155,323 ± 1804.06 UF).

Phospholipase A<sub>2</sub> activity in plasma of both species showed a positive relationship with time of incubation with the labeled substrate (*C. latirostris*:  $R^2 = 0.886$ ; *C. yacare*:  $R^2 = 0.804$ ;  $P < 0.01$ ; Fig. 4). Plasma from both caiman species exhibited PLA<sub>2</sub> activity immediately after 5 min of incubation with fluorescently labeled bacteria (*C. latirostris*: 15,897 ± 913.69 UF and *C. yacare*: 10,905 ± 893.69 UF). At all times evaluated PLA<sub>2</sub> activity in plasma of *C. latirostris* (10 min: 26,455.73 ± 729.69 UF, 15 min: 34,238.13 ± 1195.63 UF and 20 min: 39,870 ± 1356.14 UF) was higher than that of *C. yacare* (10 min: 16,934.25 ± 671.12 UF, 15 min: 21,086.25 ± 1611.14 UF and 20 min: 24,223.75 ± 1416.64 UF). Differences of 39% at 30 min (*C. yacare*, 27,022.5 ± 972.41 UF, and *C. latirostris*, 44,080 ± 1616.51 UF), 37% at 60 min (*C. yacare*, 38,782.2 ± 1575.14 UF, and *C. latirostris*, 60,677.25 ± 707.28 UF) and 27% at 120 min (*C. yacare*, 54,510.75 ± 972.87 UF, and *C. latirostris*, 75,320 ± 762.98 UF) were observed between species. Although the quantitative values of the enzyme activities were different between them, the curves shapes were similar.

Assays performed to detect the specific activity of PLA<sub>2</sub> are illustrated in Fig. 5, which represents the effects of different concentration of BPB on caiman plasma PLA<sub>2</sub> activities. Incubation of caiman plasma with labeled bacteria without BPB showed 129,297 ± 5552.93 UF for *C. latirostris*, and 131,005.78 ± 5328.9 UF for *C. yacare*; showing a progres-

sive decrease while concentration of BPB increased (*C. latirostris*, 1 mM: 17,516 ± 4327.8 UF, 5 mM: 9712 ± 5597 UF, and 10 mM: 6233 ± 4779.7 UF; *C. yacare*, 1 mM:



**Fig. 5.** Effects of p-Bromophenacyl bromide (BPB) on Caiman plasma demonstrated that the decreased in the fluorescence intensity were due to the inhibition of PLA<sub>2</sub> activity ( $P < 0.05$ ).

13,412 ± 6351.3 UF, 5 mM: 13,412 ± 6351.3 UF and 10 mM: 6589 ± 9031.2). No differences between males and females were observed in PLA<sub>2</sub> activities in any of the two species ( $P > 0.05$ ).

## DISCUSSION

Using the spectrofluorometric technique described, we detected PLA<sub>2</sub> activity in plasma of *C. latirostris* and *C. yacare*. Recently, Merchant et al. (2009) conducted a similar assay adding different proportions of bacteria without the fluorescent marker to demonstrate the specificity of the reaction. Given a fixed amount of *A. mississippiensis* serum, a reduction in fluorescent activity was observed when the amount of unlabeled bacteria in the solution increased. The catalytic activity of PLA<sub>2</sub> competed with labeled and unlabeled bacteria, reducing the amount of fluorescent product formed depending on the concentration of labeled substrate. Another study found the presence of PLA<sub>2</sub> in the serum of two species of crocodiles, *C. siamensis* and *C. porosus* (Nevalainen et al., 2009).

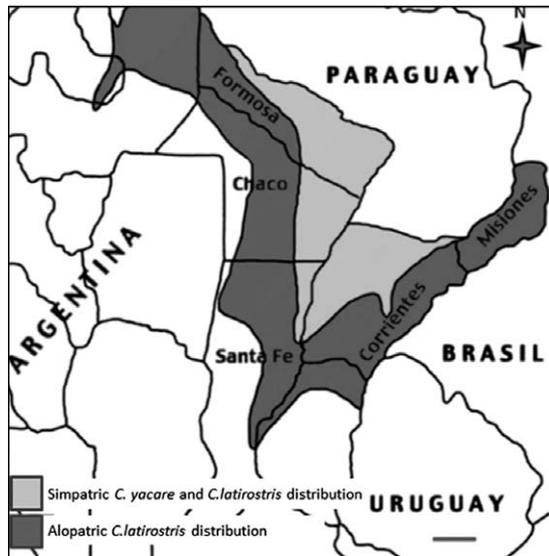
BPB has been demonstrated to be a specific inhibitor alkylating the active site of secretory an intracellular PLA<sub>2</sub> histidine residue (Volwerk et al., 1974; Longo et al., 1999). The results of the inhibition assay clearly confirmed that differences in caiman plasma fluorescent intensity with and without BPB were due to inhibition of PLA<sub>2</sub> activity. The recorded PLA<sub>2</sub> activity decreased while BPB concentration increased. It could be inferred that activity is due to the PLA<sub>2</sub> enzyme, and taking into account that only plasma was employed, it may be derived mainly from the secretory subtype. It is important to note that another enzyme with PLA<sub>2</sub>-like activity, PAF-acetylhydrolase, is not inhibited by BPB.

However, it has also been shown that this enzyme does not require Ca<sup>2+</sup> and thus would not be inhibited by PLA<sub>2</sub> (Sato et al., 1991). Therefore, any PAF-acetylhydrolase activity in the serum would have been subtracted as background activity due to the fact that the reaction would not have been stopped complexing with EDTA by the addition of Stop Buffer (Merchant et al., 2011).

Ectothermic vertebrates are considered appropriate models to assess the influence of temperature on a variety of physiological functions (Pxytycz and Zkovicz, 1994). They have been used to show that temperature plays a key role in the homeostasis of ectothermic vertebrates, including the formation of antibodies and the immune response in general (Klesius, 1990). The enzymatic activity was dependent on the incubation temperature during the reaction assay. At low temperatures (from 5 to 15°C), the enzyme activity in the plasma of *C. latirostris* was higher than in *C. yacare*. This difference may be attributable to the greater climatic tolerance of *C. latirostris* (Siroski et al., 2004).

Crocodylians have preferred body temperatures within a range of 28–33°C which is maintained by natural thermal gradients, interacting between exposure to sun, shade, hot surfaces and deep water cooler or warmer (Huchzermeyer, 2002). The maximum activity was detected at 35°C, approximately close to the caiman body temperature preference for normal physiological processes (Bassetti, 2002). As it was measured at intervals of 5°C, it is not possible to determine precisely which temperature was of the specific peak of PLA<sub>2</sub> activity.

In these species, it is possible that several enzyme activities exhibit reductions in activity at lower temperatures, and reach the highest activities at higher temperatures. As can be seen at low temperatures (less than 20°C), the plasma PLA<sub>2</sub> activity of *C. latirostris* was higher than that of *C. yacare*. These results could be related to the average temperature range in the latitudes at which these species live. *C. latirostris* has a wider geographical distribution in South America than *C. yacare* (Fig. 6). Both are distributed in northern Argentina, but the range occupied by *C. latirostris* extends much farther south than *C. yacare*. In this range, there are important differences with respect to latitudinal temperatures. The average temperature during the coldest months (June–August) in the southern limit for the range of the *C. yacare* population (30°S latitude) (Verdade, 1998), is about 17°C. In the case of *C. latirostris*, the southern distribution limit occurs at 32°S (Verdade, 1998), where the average temperature during the same months is approximately 10°C



**Fig. 6.** Caiman distribution in Argentina. *C. latirostris* distribution area covers a southernmost portion than *C. yacare* (Larriera et al., 2008).

(Servicio Meteorológico Nacional, 2010). The southernmost distribution of *C. latirostris* in Argentina is due to a greater tolerance to lower temperatures (Siroski, 2004). Although these data are not sufficient to make any kind of statement, we consider them, in addition to those previously made in our laboratory (Siroski et al., 2011) and future studies related, will allow us to elucidate if these enzyme activities may be the result of an adaptation of the immune system, along with other physiological activities, at these temperatures.

The effect of increased volumes of caiman plasma with constant amounts of the labeled bacteria solution was associated with increased PLA<sub>2</sub> activity. At the very beginning, small amounts of plasma produced a significant increase in PLA<sub>2</sub> activity, higher in *C. latirostris* plasma, which represents approximately 40% of the maximum observed. These results are consistent with similar findings in other species of crocodylians (Merchant et al., 2009; Nevalainen et al., 2009) but none of these studies detected whether the plasma contained high amounts of PLA<sub>2</sub> or increased activity when it appears in moderate amounts. This ability gives this technique an advantage, as reproducible results with very low variations can be obtained with only small volumes of plasma. In addition, future studies of PLA<sub>2</sub> activities time and concentration dependence at low reaction temperature (15°C, approximately) would be performed to contribute the controversial idea about the physiological activities adapted to geographical factors.

Another important feature of PLA<sub>2</sub> in caiman plasma is the reaction rate. Within five minutes of the exposure of bacteria to plasma, high activity was demonstrated for both species, but was higher in *C. latirostris* plasma at all times studied. These results coincide with those observed in similar studies made with dipeptidyl peptidase enzymes (DPPIV) (Siroski et al., 2011). This property was also highlighted in the work with *A. mississippiensis*, where the maximum amount of product formed was observed after 60 min of elapsed reaction and remained constant up to 120 min.

The values obtained in this work were similar to those reported in humans with severe infections, trauma, post-operative (Nevalainen et al., 2000) and bacterial sepsis (Rintala et al., 1995). Therefore, PLA<sub>2</sub> was recognized as a new acute phase protein synthesized by hepatocytes (Crowl et al., 1991; Nevalainen et al., 1996). In this case, all animals used appeared normal and healthy, and therefore, high levels of PLA<sub>2</sub> suggest that caimans have a high concentration under normal conditions, comparable to levels of human patients with inflammatory diseases (Aufenanger et al., 1993).

The enzyme PLA<sub>2</sub> is an effective antibacterial agent (Grönroos et al., 2005) and its bactericidal effects are based on the hydrolysis of phospholipids of bacteria cell membrane. These actions were confirmed to cause bacterial cell death in vitro (Grönroos et al., 2001; Koduri et al., 2002) and in vivo (Laine et al., 1999). The catalytic activity of plasma PLA<sub>2</sub> is recognized as an important effector of innate immunity (Six and Dennis, 2000; Nevalainen et al., 2009), and the high concentrations detected in the plasma of two species of caiman, could be associated with their abilities to resist the attack, or avoid infections of many different types of microorganisms (Siroski et al., 2009, 2010, 2011).

In conclusion, we suggest that the high PLA<sub>2</sub> activities in the plasma of the two species of caimans studied may be an important component of an efficient and well-developed innate immune system capable of resisting infection by some microorganisms. Powerful immunologic components have been detected in these species, and for that reason, they are excellent models to study immune phylogenetic mechanism. Several studies are developing in our laboratory detecting PLA<sub>2</sub> activity in caimans with different illnesses and others where the immune system is challenged by the infection with bacterial lipopolysaccharides. Based on the importance and multiples functions of PLA<sub>2</sub> enzymes, it should be of interest to continue increase the knowledge concerning their properties and homologies, evaluating them as a possible therapeutic application in the veterinary or even human therapeutics industry.

## ACKNOWLEDGMENTS

Authors would like to thank specially to Yacarés Santafesinos (MUPCN, Santa Fe), Yacaré Porá S.A., and IUCN-SSC Crocodile Specialist Group's Student Research Assistance Scheme (SRAS) for financial supports. We also thank to F.A. Poletta (Dirección de Investigación CEMIC-CONICET, Argentina) for statistical analysis.

## REFERENCES

- Astudillo MA, Pérez-Chacón G, Balboa MA, Balsinde J (2009) Arachidonic acid mobilization by stimuli of the innate immune response. *Inmunología* 28: 182–192
- Aufenanger J, Zimmer W, Püttmann M, Ensenauer R (1993) Determination of the catalytic activity of phospholipase A<sub>2</sub>: *E. coli*-based assay compared to a photometric micelle assay. *Eur J Clin Chem Clin Biochem* 31: 777–785
- Balsinde J, Winstead MW (2002) Phospholipase A<sub>2</sub> regulation of arachidonic acid mobilization. *FEBS Lett* 531: 2–6
- Bassetti LA (2002) Comportamento de termorregulação em jacarés-de-papo-amarelo (*Caiman latirostris*) adultos em cativeiro. Tesis de Mestrado. Esc. Super. Agri. Luiz de Queiroz, Univ. São Paulo, Piracicaba, SP, Brazil
- Budnik LT, Mukhopadhyay AK (2002) Lysophosphatidic Acid and Its Role in Reproduction. *Biol Reprod* 66: 859–865
- Burke JE, Dennis EA (2009) Phospholipase A<sub>2</sub> biochemistry. *Car-*

- diov Drugs Ther 23: 49–59
- Crowl RM, Stoller TJ, Conroy RR, Stoner CR (1991) Induction of phospholipase A<sub>2</sub> gene expression in human hepatoma cells by mediators of the acute phase response. *J Biol Chem* 266: 2647–2651
- Dawson B, Trapp RG (2001) *Basic & Clinical Biostatistics*. 3<sup>rd</sup> ed., The McGraw-Hill Companies, Inc., NY
- Fry BG, Vidal N, Norman JA, Vonk FJ, Scheib H, Ramjan SFR, et al. (2006) Early evolution of the venom system in lizards and snakes. *Nature* 439: 584–588
- Funk CD (2001) Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* 294: 1871–1875
- Grönroos JO, Laine VJO, Janssen MJW, Egmond MR, Nevalainen TJ (2001) Bactericidal properties of group IIA and V phospholipases A<sub>2</sub>. *J Immunol* 166: 4029–4034
- Grönroos JO, Salonen JH, Viander M, Lambris JD, Nevalainen TJ, Laine VJO (2005) Roles of group IIA phospholipase A<sub>2</sub> and complement in killing of bacteria by acute phase serum. *Scandinavian Journal of Immunology* 62: 413–419
- Huchzermeyer FW (2002) Diseases of farmed crocodiles and ostriches. *Rev Sci Tech* 21: 273–274
- Kini RM (2003) Excitement ahead: structure, function and mechanism of snake venom phospholipase A<sub>2</sub> enzymes. *Toxicon* 42: 827–840
- Klesius PH (1990) Effect of size and temperature on the quantity of immunoglobulin in channel catfish, *Ictalurus punctatus*. *Vet Immunol Immunopathol* 24: 187–195
- Koduri RS, Grönroos JO, Laine VJO, le Calvez C, Lambeau G, Nevalainen TJ, Gelb MH (2002) Bactericidal properties of human and murine groups I, II, V, X and XII secreted phospholipases A<sub>2</sub>. *J Biol Chem* 277: 5849–5857
- Kudo I, Murakami M (2002) Phospholipase A<sub>2</sub> enzymes. *Prostaglandins & Other Lipid Mediators* 68: 3–58
- Laine VJO, Grass DS, Nevalainen TJ (1999) Protection by group II Phospholipase A<sub>2</sub> against *Staphylococcus aureus*. *J Immunol* 162: 7402–7408
- Lance VA, Lauren D (1984) Circadian variation in plasma corticosterone in the American alligator, *Alligator mississippiensis*, and the effects of ACTH injections. *Gen Comp Endocrinol* 54: 1–7
- Larriera A, Imhof A, Siroski P (2008) Estado actual de los programas de conservación y manejo del género Caiman en Argentina. In “Contribución al Conocimiento del Género Caiman de Suramerica” Ed by J Castroviejo, J Ayarzagüena, A Velasco, Public. Asoc. Amigos de Doña Ana, Sevilla, España, pp 143–179
- Longo WE, Grossman EM, Erickson B, Panesar N, Mazuki AE, Kaminski DL (1999) The effect of phospholipase A<sub>2</sub> inhibitors on proliferation and apoptosis of murine intestinal cells. *J Surg Res* 84: 51–56
- Mansueto P, Vitale G, Cascio A, Seidita A, Pepe I, Carroccio A, et al. (2012) New insight into immunity and immunopathology of rickettsial diseases. *Clin Develop Immunol* 2012: ID 967852
- Merchant ME, Heard R, Monroe C (2009) Characterization of phospholipase A<sub>2</sub> activity in serum of the American alligator (*Alligator mississippiensis*). *J Exp Zool A* 311: 662–666
- Merchant M, Juneau K, Gemillion J, Falconi R, Doucet A, Shirley MH (2011) Characterization of serum phospholipase A<sub>2</sub> activity in three diverse species of West African crocodiles. *Biochem Res Int* 2011: ID 925012, doi:10.1155/2011/925012
- Murakami M, Nakatani Y, Atsumi G, Inoue K, Kudo I (1997) Regulatory functions of phospholipase A<sub>2</sub>. *Crit Rev Immunol* 17: 225–283
- Nevalainen TJ, Kallajoki M, Pesonen E, Andersson S, Kärkkäinen P, Höckerstedt K (1996) Origin of circulating group II phospholipase A<sub>2</sub> in hepatocytes in a patient with epitheloid hemangioendothelioma of the liver. *Lab Invest* 74: 585–591
- Nevalainen TJ, Haapamäki MM, Grönroos JM (2000) Roles of secretory phospholipases A<sub>2</sub> in inflammatory diseases and trauma. *Bioch Biophys Acta* 1488: 83–90
- Nevalainen TJ, Kanchanapangka S, Youngprapakorn P, Webb GJW, Manolis SC, Scott KF (2009) Phospholipase A<sub>2</sub> activity of crocodile serum. *Amphibia-Reptilia* 30: 119–125
- Perrin-Cocon L, Agaugué S, Coutant F, Masurel A, Bezzine S, Lambeau G, et al. (2004) Secretory phospholipase A<sub>2</sub> induces dendritic cell maturation. *Eur J Immunol* 34: 2293–2302
- Pxytycz B, Zkovicz AJ (1994) Differential effects of temperature on macrophages of ectothermic vertebrates. *J Leuk Biol* 56: 729–731
- Rintala E, Pulkki K, Mertsola J, Nevalainen T, Nikoskelainen J (1995) Endotoxin, interleukin-6 and phospholipase-A<sub>2</sub> as markers of sepsis in patients with hematological malignancies. *Scand J Infect Dis* 27: 39–43
- Satoh K, Imaizumi T, Kawamura Y (1991) Platelet-activating factor (PAF) stimulates the production of PAF acetylhydrolase by the human hepatoma cell line, HepG2. *J Clin Invest* 87: 476–481
- Shrestha S, Kim Y (2009) Biochemical characteristics of immune-associated phospholipase A<sub>2</sub> and its inhibition by an immunopathogenic bacterium, *Xenorhabdus nematophila*. *J Microbiol* 47: 774–782
- Silliman CC, Moore EE (2002) Presence of the M-type sPLA (2) receptor on neutrophils and its role in elastase release and adhesion. *Am J Physiol Cell Physiol* 283: 1102–1113
- Siroski PA (2004) *Caiman latirostris* and *Caiman yacare* Population Surveys in Formosa Province, Argentina in Crocodiles. Proceeding of the 17th Working Meeting of the Crocodile Specialist Group of the Species Survival Commission of IUCN, The World Conservation Union, Darwin, NT, Australia, pp 443–446
- Siroski PA, Piña CI, Larriera A, Merchant ME, Di Conza J (2009) Plasma activity of Broad-snouted Caiman (*Caiman latirostris*). *Zool Stud* 48: 238–242
- Siroski PA, Merchant ME, Parachú Marcó MV, Piña CI, Ortega HH (2010) Characterization of serum complement activity of broad-snouted caiman (*Caiman latirostris*, Crocodylia: Alligatoridae). *Zool Stud* 49: 64–70
- Siroski PA, Merchant ME, Parachú Marcó MV, Poletta GL, Ortega HH (2011) Comparison of plasma dipeptidyl peptidase IV activity in two caiman species: *Caiman latirostris* and *Caiman yacare* (Crocodylia, Alligatoridae). *Anim Biol* 61: 199–210
- Six DA, Dennis EA (2000) The expanding superfamily of phospholipase A<sub>2</sub> enzymes: classification and characterization. *Bioch Biophys Acta* 1488: 1–19
- Turton JA, Ladds PW, Manolis SC, Webb GJW (1997) Relationship of blood corticosterone, immunoglobulin and haematological values in young crocodiles (*Crocodylus porosus*) to water temperature, clutch of origin and body weight. *Aust Vet J* 75: 114–119
- Vandermeers A, Vandermeers-Piret MC, Vigneron L, Rathe J, Stievenart M, Christophe J (1991) Differences in primary structure among five phospholipases A<sub>2</sub> from *Heloderma suspectum*. *Eur J Biochem* 196: 537–544
- Verdade LM (1998) *Caiman latirostris*. In “Crocodiles: Status Survey and Conservation Action Plan” Ed by JP Ross, IUCN—The World Conservation Union, Gland, Switzerland, pp 18–20
- Volwerk JJ, Pieterse WA, de Haas GH (1974) Histidine at the active site of phospholipase. *Biochemistry* 13: 1446–1454
- Webb GJW, Manolis SC (1983) *Crocodylus johnstoni* in the MacKinnlay River, N.T.V. Abnormalities and injuries. *Aust Wildl Res* 10: 407–409
- Webb GJW, Messel H (1977) Abnormalities and injuries in the estuarine crocodile, *Crocodylus porosus*. *Aust Wildl Res* 4: 311–319
- Zippel KC, Lillywhite HB, Mladnich CR (2003) Anatomy of the crocodilian spinal vein. *J Morphol* 258: 327–335

(Received April 19, 2012 / Accepted July 24, 2012)