Characterization of serum dipeptidyl peptidase IV activity in three diverse species of West African crocodilians

Mark Merchant[1], Arian Royer[1], Quintin Broussard[1], Sarah Gilbert[1], Rodolfo Falconi[1] & Matthew H. Shirley[2]

[1]McNeese State University, Department of Chemistry, Lake Charles, Louisiana, USA
[2]University of Florida, Department of Wildlife Ecology and Conservation, Gainesville, Florida, USA

Serum dipeptidyl peptidase IV (DPPIV) activity was characterized in three divergent and sympatric species of West African crocodiles. The serum of the Nile crocodile (*Crocodylus niloticus*) exhibited higher DPPIV activity than that of the African dwarf crocodile (*Osteolaemus tetraspis*) and the slender-snouted crocodile (*Mecistops cataphractus*). Kinetic analyses showed that the rate of product formation was higher in serum of *C. niloticus* with respect to time, and it was confirmed by double reciprocal plot analysis that the V_{max} for serum of *C. niloticus* was higher than the other two species. However, the Michaelis constants were very similar for all three species, indicating that the *C. niloticus* DPPIV enzyme may be a more efficient catalyst. Thermal activity profiles demonstrated that the serum DPPIV activities of all three species increased substantially with temperature. Although activity of *C. niloticus* was higher than that of *O. tetraspis* and *M. cataphractus* at all temperatures investigated, linear increases of activity with temperature were noted for all three species. The results from this study show that three diverse species of West African crocodilians express soluble serum DPPIV.

Key words: Crocodylus niloticus, DPPIV, innate immunity, *Mecistops cataphractus*, *Osteolaemus tetraspis*, reptilian, T-cell activation

INTRODUCTION

The Nile crocodile (*Crocodylus niloticus*) is a true crocodile (i.e. genus *Crocodylus*) and is one of the most studied of the crocodilians. It is a generalist in virtually all of its habits, including both foraging and habitat occupancy, though its breeding requirements prevent significant occupation of forested areas (Cott, 1961; Shirley et al., 2009; Fergusson, 2010). The Nile crocodile is a highly social species with well-established breeding and dominance hierarchies, and aggressive social interactions often result in extensive injury (Cott, 1961; Modha, 1967). Recent investigations into the systematics of this species have revealed the presence of a divergent, cryptic lineage within what is currently known as *C. niloticus*, though the available evidence supports the notion that the nominate lineage is distributed throughout coastal central Africa (Schmitz et al., 2003).

The slender-snouted crocodile (*Mecistops cataphractus*) is considered the least known crocodilian in the world (Shirley, 2010; Eaton, 2010), and is thought to be part of an older sister lineage to the true crocodiles (Brochu, 2003). Once considered a member of the genus *Crocodylus*, *M. cataphractus* has recently been placed in its own monotypic genus (McAliley et al., 2006). It is a medium-bodied species that inhabits the forested wetlands of central and western Africa. As its common name suggests, it is a longirostrine species adapted for the speed necessary for a highly aquatic, piscivorous lifestyle (Waitkuwait, 1985, 1989; Shirley, 2010). Very little is known of its basic ecology, though recent observations suggest that, despite being among the most vocal crocodile species, they lead very solitary lifestyles (Shirley, pers. obs.).

The African dwarf crocodile (*Osteolaemus tetraspis*) is a diminutive species found throughout the forests of central and western Africa (Pauwels et al., 2006). It is among the most terrestrial of crocodilians and can be encountered in the forest kilometres from water (Waitkuwait, 1985). The skin of *O. tetraspis* is heavily ossified, an adaptation attributed to both its terrestrial nature (as a defence mechanism against terrestrial predators) and its extensive use of burrows (Waitkuwait, 1985, 1989; Eaton, 2010). A recent study has proposed that this taxon is actually comprised of three distinct lineages divergent at the species level (Eaton et al., 2009). The nominate lineage is found in western Central Africa.

In western Central Africa, these three, highly divergent crocodiles can be found sympatrically, though with some degree of habitat partitioning. In Gabon, *O. tetraspis* is the most widely distributed crocodile, and can be found throughout due to the abundance of forest and swamp habitat (Pauwels et al., 2007). *Mecistops cataphractus* is equally widely distributed, though it is limited to the larger forested river and wetland habitats. For reasons as yet unexplained, it does not use the expansive coastal lagoon network extensively (Pauwels & Vandeweghe, 2008). The current distribution of *C. niloticus* is limited to coastal lagoons surrounded by savannah–forest mosaic, including

Correspondence: Mark Merchant, Department of Chemistry, 450 Beauregard, Kirkman Hall 221A, McNeese State University, Lake Charles, LA 70609, USA. *E-mail:* mmerchant@mcneese.edu

frequent use of the marine environment for dispersal and seasonal movements (Pauwels & Vandeweghe, 2008).

Dipeptidyl peptidase IV (DPPIV) is a ubiquitous enzyme that catalyses the proteolysis of N-terminal L-alanine or L-proline residues (Chen, 2006). DPPIV activity has been reported to exhibit extensive immune activity (Aytac & Dang, 2004), is required for T-cell immune responses in mammals (Morimoto & Schlossman, 1998), and some of the natural substrates of DPPIV are involved in immunomodulation (Boonacker & Van Noorden, 2003). DPPIV activity is linked to T-cell costimulatory efficacy (Tanaka et al., 1993, 1994) and has been correlated with immunocompetence *in vivo* (Vanham et al., 1993). It has also been reported that DPPIV activity is required for full T-cell activation *in vivo* (Geppert et al., 1990; Kameoka et al., 1993; Reinhold et al., 2003), and has been linked to growth regulation of lymphocytes (Ansorge et al., 1997). Most of the studies of DPPIV activity have been conducted using mammalian animal models, and there is currently limited knowledge concerning reptilian DPPIV. The majority of reptilian DPPIV studies have been limited to snake venoms (Aird, 2008; Ogawa et al., 2006). However, recent studies have focused on the characterization of serum DPPIV activity in the American alligator (*Alligator mississippiensis*, Merchant et al., 2009a), American crocodile (*Crocodylus acutus*, Merchant et al., 2010) and the broad-snouted (*Caiman latriostris*) and yacare (*Caiman yacare*, Siroski et al., 2010) caiman. Since crocodilians have been shown to exhibit advanced innate immunity (Merchant et al., 2003), and because DPPIV is proposed to have extensive immune function, this study was undertaken to determine the activity of DPPIV in these three diverse West African crocodilians. The current study is a comparison of the serum DPPIV activity of *C. niloticus*, *M. cataphractus* and *O. teraspis*, and is discussed with respect to other crocodilian species for which this enzyme activity has been described.

MATERIALS AND METHODS

Chemicals and biochemicals. Pro-Ala-AFC was purchased from Anaspec (San Jose, CA, USA). Diprotin A (Leu-Pro-Leu), Tris-HCl, $CaCl_2$ and EDTA were purchased from Sigma Aldrich (St Louis, MO, USA).

Treatment of animals. Crocodiles were captured from the N'Doudou Lagoon, Bongo River and Nyanga River areas of the Gamba Complex of the central coast of Gabon, Africa. Animals were captured at night, with the aid of a spotlight, using tongs or cable-locking snares, and a few larger animals were captured using small harpoons. Blood was collected from crocodiles via the spinal vein (Olsen et al., 1977; Zippel et al., 2003). All of the activities involving handling of the animals used in this study were approved by McNeese State University and University of Florida Animal Care and Use Committees.

DPPIV assay. DPPIV activity was determined using a dipeptide (Ala-Pro) linked to 7-amino-4-trifluoromethyl coumarin (AFC) via an amide linkage. The cleavage of AFC away from the dipeptide resulted in a substantial increase in fluorescent activity. For the experiment in which the influence of serum titre on fluorescent activity was observed, different volumes (0–500 μL) of crocodilian serum were diluted to 750 μL of assay buffer (100 mM Tris, pH 7.4, with 2 mM $CaCl_2$) and incubated with 10 μL of 1.0 mg/mL Ala-Pro-AFC substrate for 60 min at ambient temperature. The reaction was halted by the addition of 750 mL of stop buffer (100 mM Tris, pH 7.4, with 20 mM EDTA). The samples were transferred to cuvettes and the fluorescent intensities were measured at an excitation λ of 395 nm (slit=2 nm) and an emission λ of 530 nm (slit=2 nm).

For the experiment in which the rate of product formation was determined, 2.0 mL of crocodilian serum was added to 27.7 mL of assay buffer. Upon the addition of 400 μL of 1.0 mg/mL Ala-Pro-AFC substrate, aliquots (750 μL) were removed to 750 μL of stop buffer at different time points, the samples were transferred to cuvettes and fluorescent intensities were measured as described above.

To determine the Michaelis–Menten character of the DPPIV activities of each crocodilian species, the assay was conducted in the presence of different concentrations (0–838 μM) of Ala-Pro-AFC substrate. The reaction was allowed to proceed for 60 min at ambient temperature, and the reaction was concluded by the addition of 750 mL of stop buffer. The samples were then transferred to cuvettes and fluorescent intensities were measured as described above.

Crocodilian serum DPPIV samples were assayed at various temperatures (5–40 °C) to determine the thermodynamic characteristics of their activities. Serum (50 μL) was incubated with 700 uL of assay buffer and 10 uL of 1.0 mg/mL Ala-Pro-AFC. The reaction was allowed to proceed for 60 min, and was halted by the addition of 750 μL of stop buffer. The fluorescent activity of each replicate was then determined as described above.

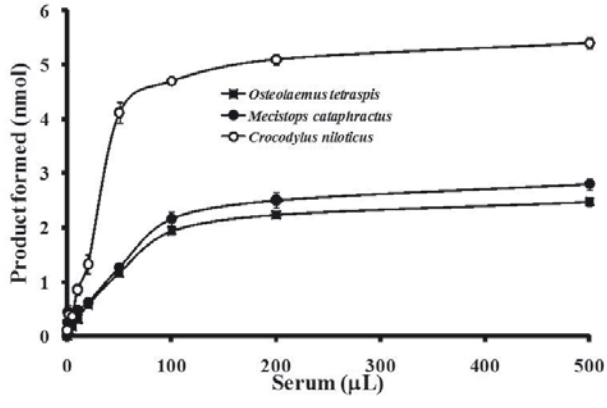

Fig. 1. Serum titre-dependent DPPIV activity in three species of West African crocodilians. Different volumes of serum were incubated with Ala-Pro-AFC substrate, and the AFC proteolytic product was measured fluorimetrically. The results are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations.

The specificity of the cleavage of AFC from the Ala-Pro dipeptide was determined by incubation with diprotin A, a specific inhibitor of DPPIV activity (Rahfeld et al., 1991). Serum (50 µL) was incubated with 650 uL of assay buffer, several concentrations (0–1.5 mM, final concentration) of diprotin A and 10 uL of 1.0 mg/mL Ala-Pro-AFC. The reaction was allowed to continue for 60 min before 750 µL of stop buffer were added, and the fluorescent activity of each reaction was then determined as described above.

Statistics and controls. The DPPIV activities are expressed as nmol of product formed and represent the means ± standard deviations from four independent analytical determinations. The fluorescent intensities derived from each assay were compared to a linear standard curve generated with the AFC enzymatic product, and the mass of product formed was calculated using the linear regression relation derived from the data (R=0.998, data not shown). The statistical comparisons between groups were conducted using analyses of variance and Duncan's post-hoc comparisons and $P<0.05$ was chosen as the standard probability of statistical significance.

RESULTS

Significant DPPIV activity was measured in only one µL of serum from *C. niloticus* (0.45±0.12 nmol), *M. cataphractus* (0.26±0.03 nmol) and *O. tetraspis* (0.07±0.01 nmol) (Fig. 1). The product formation in the presence of low serum volumes (0–50 µL) of all three crocodilian species increased with serum titre. However, serum of *C. niloticus* produced a sharper increase ($P<0.05$) in activity than serum of *M. cataphractus* or *O. tetraspis*. In addition, the maximum product formation activity for serum of *C. niloticus* (5.42±0.11 nmol) was much higher ($P<0.01$) than serum of *M. cataphractus* (2.81±0.10 nmol) or *O. tetraspis* (2.48±0.07 nmol). All three species exhibited a near linear increase in product accumulation up to at least 50 µL of serum, and near maximal levels were achieved by the inclusion of 100 µL of serum.

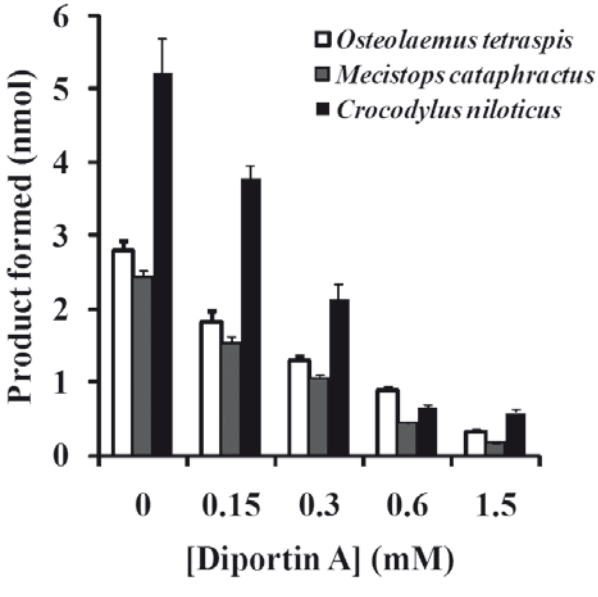

Fig. 2. Concentration-dependent inhibition of DPPIV activity by diprotin A. Crocodilian DPPIV was assayed in the absence, and in the presence of different concentrations of diprotin A, a specific DPPIV inhibitor. The results are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations.

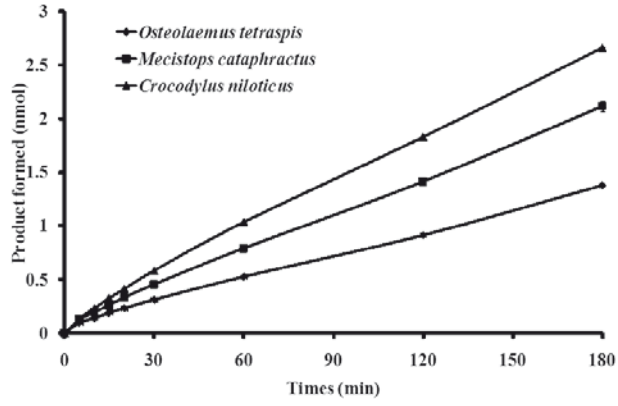

Fig. 3. Time-dependent African crocodilian serum DPPIV activity. Crocodilian serum was incubated with Ala-Pro-AFC for different amounts of time, and the fluorescent product quantified. The results are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations.

Inclusion of 0.15, 0.3, 0.6, or 1.5 mM diprotin A (Leu-Pro-Leu), a specific DPPIV inhibitor, resulted in a 27.5, 59.3, 87.4 or 88.7% decrease ($P<0.05$ for all, relative to control) in DPPIV activity of serum from *C. niloticus*, respectively, relative to activity in the absence of inhibitor (Fig. 2). The same concentrations of diprotin A caused similar decreases ($P<0.05$) in DPPIV activities in serum of *O. tetraspis and M. cataphractus* as well.

The kinetic activities of African crocodile DPPIV are illustrated in Figure 3. The accumulation of fluorescent product was higher for serum of *C. niloticus* ($P<0.05$) than for *O. tetraspis* or *M. cataphractus*. The serum DPPIV from each of the three crocodilian species exhibited linear enzyme activity with respect to time. The slope of the lines, 1.48×10^{-2}, 1.17×10^{-2} and 0.77×10^{-2} for *C. niloticus*, *M. cataphractus* and *O. tetraspis*, respectively, indicated that serum DPPIV of *C. niloticus* was more active ($P<0.01$) than that of *M. cataphractus* and *O. tetraspis*, respectively.

The serum DPPIV activities for all three crocodilians followed Michaelis–Menten kinetics (Fig. 4). The serum DPPIV activities for all three crocodilians followed Michaelis–Menten kinetics. The K_m values were similar for all three species (*C. niloticus* = 0.17, *M. cataphractus* = 0.17 and *O. tetraspis* = 0.18 nmol/min), exhibiting only a 5.9% difference among the activities. However, the V_{max} values for the enzymes reflected large ($P<0.01$)

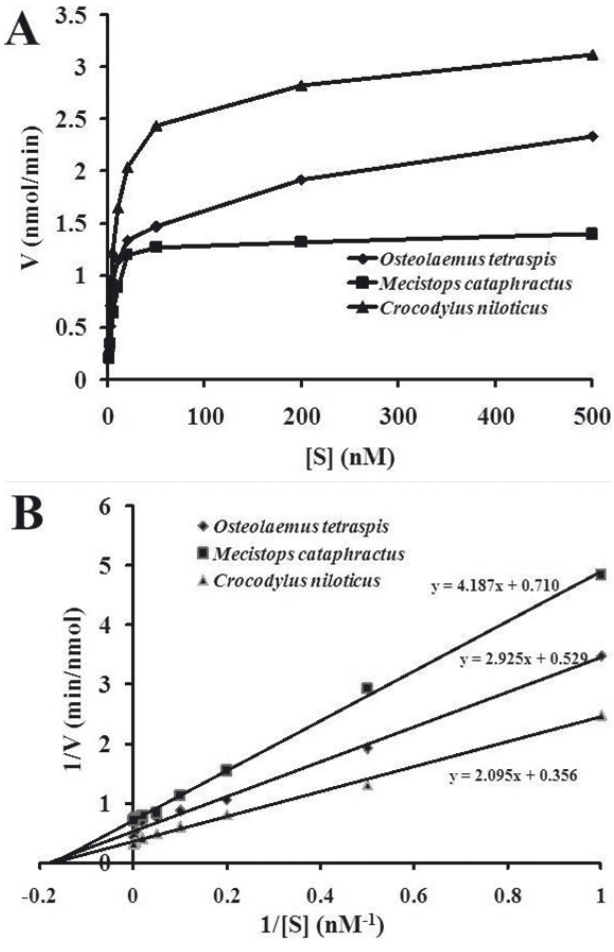

Fig. 4. Dependence of DPPIV activity of *C. niloticus*, *O. tetraspis* and *M. cataphractus* on substrate concentration. A) Different concentrations of Ala-Pro-AFC substrate were incubated with crocodile serum, and the DPPIV activity was measured spectrofluorimeterically. B) Double reciprocal plot analysis allowed for the determination of V_{max} and K_m values for each species. Each data point represents the result of four independent determinations. V_{max} and K_m calculations were determined by linear regression analyses of the means of four determinations for each data point.

differences of up to 50.2% (*C. niloticus* = 2.81 nM, *M. cataphractus* = 1.41 nM and *O. tetraspis* = 1.89 nmol).

The serum DPPIV activities of all three species showed a strong, positive correlation with increasing temperature (Fig. 5), although serum from *C. niloticus* exhibited higher product formation ($P<0.05$) than *M. cataphractus* and *O. tetraspis* sera at all temperatures tested. Serum DPPIV activities from *C. niloticus* were approximately 60% higher than those displayed by *M. cataphractus* and *O. tetraspis* over the entire temperature range tested. The serum DPPIV activities in samples from *M. cataphractus* and *O. tetraspis* were not statistically different from one another ($P>0.05$) at any temperature tested. The DPPIV activities for all three crocodilian species were approximately three-fold higher at 40 °C than at 5 °C.

DISCUSSION

The activities observed in these three African species (Fig. 1) are similar to that measured in *C. acutus* (Merchant et al., 2010), but approximately twenty-fold lower than that measured in *A. mississippiensis* (Merchant et al., 2009a), *C. latirostris* and *C. yacare* (Siroski et al., 2010). The accumulation of DPPIV enzymatic product occurred much more rapidly for serum of *C. niloticus* than for *M. cataphractus* and *O. tetraspis*, at the same serum titres. These data are confirmed by the results of the kinetic accumulation of product (Fig. 3). The serum DPPIV of *C. niloticus* was 26.5 and 92.2% more active ($P<0.01$) than that of *M. cataphractus* and *O. tetraspis*, respectively, at the same serum titres. The slopes of the lines, derived by linear regression analyses, are indicative of the rates of product formation. This higher slope could be due to a higher concentration of serum DPPIV in *C. niloticus*. However, the data in Figure 4 show that the V_{max} was similar but the K_m for DPPIV of *C. niloticus* was higher than that of the other two species. This could be an indication that the serum DPPIV of *C. niloticus* displays more efficient product formation, at the same substrate and enzyme concentration, than that of *M. cataphractus* or *O. tetraspis*. Although the V_{max} values are the same, indicating that the maximum rate of product formation (at high substrate concentrations) is similar for all three species, the serum DPPIV of *C. niloticus* generates more product in the presence of low substrate concentrations, which may be due to higher substrate turnover efficiency. However, this is difficult to determine, because the serum DPPIV enzyme concentration for each species cannot be ascertained at this time, and thus the K_{cat} values for each species cannot be calculated.

Vertebrates express a broad spectrum of serum proteases that serve a wide variety of physiological functions (Hedstrom, 2002; Chapman et al., 1997). When measuring the activity of specific proteases, it is important to use inhibitors to ensure that the activities observed are due to the presence of the enzyme of interest. Diprotin A, a specific inhibitor of DPPIV enzyme activity, was used to show the specificity of the proteolytic assay used to detect this enzyme activity in the crocodilian sera samples (Fig. 2). The serum DPPIV activities of all three West African crocodiles were inhibited, in a concentration-dependent manner, by diprotin A. Since diprotin A is known to be a specific inhibitor of DPPIV activity (Leiting et al., 2003), the effects of diprotin A on these activities indicates that the proteolytic activities are likely to be due to the presence of DPPIV.

Incubation of serum of *C. niloticus* resulted in an increased accumulation of product, relative to *M. cataphractus* and *O. tetraspis*, at the same titres. This could be due to increased circulating concentrations of DPPIV in *C. niloticus*, or a more active enzyme with higher substrate turnover. The serum DPPIV levels reported here for West African crocodiles are nearly a hundred times lower

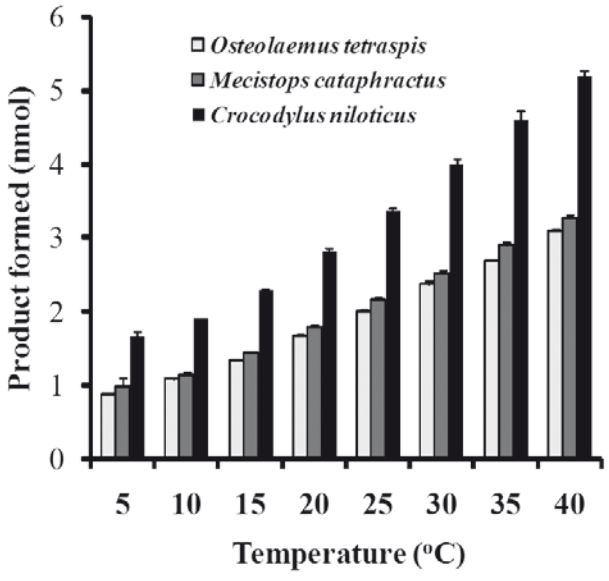

Fig. 5. Temperature-dependence of serum DPPIV in three West African crocodilian species. DPPIV in serum samples from *C. niloticus*, *O. tetraspis* and *M. cataphractus* were determined at different temperatures. The results are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations.

than those reported for *A. mississipiensis* (Merchant et al., 2009a) and *C. latirostris* and *C. yacare* (Siroski et al., 2010). To date, it is not known whether this reflects differences in DPPIV expression between new world and old world crocodilians, alligatorids versus crocodylids, or is simply coincidence. Further studies will focus on the comparisons of serum DPPIV activities in crocodylid, alligatorid and gavialid species. Although the West African crocodilian DPPIV activities were much lower than those measured in other crocodilian species, these serum DPPIV levels are comparable to those measured in human serum (Varona et al., 2010).

The serum DPPIV activity in all three West African crocodilian species showed a strong correlation with temperature (Fig. 5). The increase in activity with temperature exhibited an almost linear correlation, increasing throughout the entire thermal range (5–40 °C). However, it is interesting to note that many metabolic and physiological functions in crocodilian species are optimal near 30–32 °C (Lance, 1994). This is the target temperature range to which most crocodilians thermoregulate internal body temperatures (Seebacher & Grigg, 1997; Seebacher et al., 1999, 2003). In addition, serum DPPIV from *C. acutus* (Merchant et al., 2010), *C. latirostris* and *C. yacare* (Siroski et al., 2010) and *A. mississippiensis* (Merchant et al., 2009a) also increased from 30–40 °C. However, it is interesting to note that other important enzyme-dependent immune parameters in *A. mississippiensis*, such as serum complement activity, are optimal near 30 °C and decrease at 35 and 40 °C (Merchant et al., 2005). The increased DPPIV activity observed in crocodilians at higher temperatures may indicate an immune system adaptation. It is known that elevated temperatures can increase immune response efficiencies in snakes (Deakins, 1980). In addition, the physiological effects of bacterial and viral infections in crocodilians can be intensified by maintaining reptiles in artificially low thermal environments (Jacobsen, 1980; Kleese, 1980; Marcus, 1980). Furthermore, *A. mississipiensis* exposed to bacterial LPS can generate a behavioural febrile response (Merchant et al., 2007). Specimens of *C. latirostris* with acute injuries resulting from territorially aggressive behaviours have been observed to bask much longer than other animals in the same pool (Carlos Piña, unpublished observations). Since it is known that DPPIV plays important roles in immunity (Aytac et al., 2004), the increase in crocodilian internal body temperature during an infection may allow for increased activity of serum DPPIV, and thus increased activation of T-cells (Geppert et al., 1990; Kameoka et al., 1993; Reinhold et al., 2003) and lymphocyte proliferation (Ansorge, 1997). Other crocodilian enzymes with associated immune function have been shown to increase at elevated temperatures (Merchant et al., 2009b), thus strengthening the argument for the potential role of increased immunity during behavioural febrile responses.

The results from this study show that three diverse species of West African crocodilians express soluble serum DPPIV. Higher activity was exhibited in *C. niloticus* serum than *M. cataphractus* or *O. tetraspis*. Although DPPIV has been linked to a variety of immune events in mammalian systems, this study does not link crocodilian DPPIV to functional immunity. Future studies will focus on the role of DPPIV in crocodilian immunity.

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