Asian Herpetological Research 2013, 4(1): 22–27
DOI: 10.3724/SP.J.1245.2013.00022

Effects of Different Rearing Strategies and Ages on Levels of Natural Antibodies in Saliva of the Philippine Crocodile

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Abstract The endemic Philippine crocodile (*Crocodylus mindorensis*) is a relatively small, critically endangered freshwater crocodile. In a head start program, crocodile hatchlings are caught in the wild, reared in captivity, and released back into the wild after two years. The current study aimed to determine optimal rearing strategies of Philippine crocodile hatchlings, including identification of possible diseases during rearing, and studying the effect of ages on natural antibody (NAb) levels. Thirty Philippine crocodiles were divided into two groups, half were reared with a hiding board, and half without the hiding board. Both groups received three different kinds of diets: meat, shrimp, or a combination of both. Saliva samples of the crocodiles were taken three times over a period of three months to test for NAb levels. Saliva samples were also taken from older crocodiles and crocodiles from different locations. NAb titres were compared to sheep red blood cells. Each time saliva samples were taken, a health check was done. The results showed that crocodiles would prefer the hiding board, and neither housing nor diet could affect the level of NAb titres in saliva. A positive correlation was found between NAb titres and body size, weight and age. Wild hatchlings had higher NAb titres than the hatchlings born in captivity, but the difference diminished with ageing. Five different diseases were found.

Keywords behaviour, *Crocodylus mindorensis*, diet, disease, husbandry, stress

1. Introduction

The Philippine crocodile (*Crocodylus mindorensis*; Schmidt, 1935) is a relatively small freshwater crocodile, endemic to the Philippines. It is listed as critically endangered (IUCN, 2012) as a result of hunting and habitat loss (Van Weerd, 2010). The Mabuwaya Foundation implements a conservation program for one of the last remaining reproducing wild Philippine crocodile populations in Luzon (van Weerd, 2010). A head start strategy has been implemented where wild Philippine crocodile hatchlings are reared in the Municipal Philippine Crocodile Rearing Station (MPCRS), in San Mariano (van Weerd and van der Ploeg, 2008) for 18 to 24 months. Later, the young crocodiles are released back into their natural habitat, increasing survival rates to 72% compared to 0%–47% per nest in the wild (van de Ven *et al.*, 2009).

When hatchlings are reared in captivity, husbandry-related stress is a problem. Various conditions of stress (such as cold, overheating, overcrowding, handling, lack of hiding places and disturbance) can compromise the immune system, which affects growth performance, and also causes diseases and mortality in hatchlings (Foggin, 1987; Huchzermeyer *et al*., 1994; Huchzermeyer, 2003). The small surviving number of wild Philippine crocodiles are potentially susceptible to diseases, perhaps exacerbated by inbreeding, although there is no existing information on diseases of Philippine crocodiles. Multiple diseases are found in other wild and captive crocodile species, such as crocodile pox (Gerdes, 1991), chronic stress dermatitis (Huchzermeyer, 2003), an osteomalacia or "rubber jaw" (Huchzermeyer, 1986) and different fungal skin conditions are also known, caused by *Aspergillus*,

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Received: 7 December 2012 Accepted: 21 February 2013

Penicillium and *Curvularia* spp. (Buenviaje *et al.*, 1994).

Humoral immune response, important to prevent and fight pathogens, in reptiles is similar to mammalian, albeit slower, and does not increase in titre and binding affinity upon second exposure (Zimmerman *et al.*, 2009). Due to relatively slow and less robust adaptive humoral responses, reptiles may rely more on pre-existing barriers such as natural antibodies (NAbs; Zimmerman *et al.*, 2009). NAbs act as a first line of defence against pathogens such as bacteria and viruses (Ochsenbein *et al.*, 1999; Ochsenbein and Zinkernagel, 2000), play an important role in activating immune responses against infections (Ochsenbein *et al.*, 1999; Zhou and Notkins, 2004), and are involved in the regulation of B and T cells, inflammation, and cytokine action (Baker and Ehrenstein, 2002; Bruley-Rosset *et al.*, 2003). Though NAbs perform important immune functions, they are often regarded as 'nonspecific background', and are, therefore, regularly ignored in immunological studies (Madsen *et al.*, 2007; Ujvari and Madsen, 2011). NAbs were found in reptiles such as alligators (Longenecker and Mosmann, 1980), water pythons (Madsen *et al.*, 2007) and garter snakes (Sparkman and Palacios, 2009).

Immune functions can deteriorate with age (immunosenescence), as found in mammals (Hayward *et al.*, 2009; Pelletier *et al.*, 2005), birds (Cichon *et al.*, 2003; Palacio *et al.,* 2007; Saino *et al.*, 2003) and snakes (Madsen and Ujvari, 2006). In humans, it results in increased mortality and morbidity (Miller, 1996; Pawelec *et al.*, 2010) and may restrict longevity (Ujvari and Madsen, 2011). However, the levels of NAbs increase with aging (Attansio *et al.*, 2001; Berghof *et al.*, 2010; Madsen *et al.* 2007; Nobrega *et al.*, 1996; Parmentier *et al.*, 2004). In fish and poultry, high NAb titres were related with increasing survival (Kachamakova *et al.*, 2006; Star *et al.*, 2007) and may thus be an indicator of enhanced survival. The aims of this study were to identify the impact of various rearing factors and different ages on NAb levels and on possible diseases in captive Philippine crocodiles.

2. Material and Methods

2.1 Subjects and samples Thirty hatchling crocodiles were captured from the wild and brought to the MPCRS. Saliva samples were taken by swiping a cotton bud 3 times over the tongue while the crocodile was constrained. Samples were collected 3 times at the start of the experiment, after 4 weeks and after 8 weeks. Samples were taken from crocodiles of two different age classes. The first age class (n = 24) was ca. 6 weeks old at the start of the experiment with an average total length (ATL) of 30.3 ± 0.7 cm. The animals originated from two different nests; Dadugen Lake (n = 18) and Dinang Creek (n = 6). The second age class (n = 6) was ca. 14 months old at the start of the experiment with an ATL of 41.9 ± 2.4 cm, and they originated from 3 different nests: Dadugen Lake (n = 1), Gilingan (n = 3) and Disulap River (n = 2).

For the second part of the study, a single saliva sample was taken once from the 30 crocodiles mentioned above, from 36 other crocodiles at MPCRS and from 31 Philippine crocodiles in zoos. Of the 36 crocodiles at MPCRS, 20 were ca. 14 months old juveniles (41.9 ± 2.4 cm ATL) and 16 were juveniles between 26–62 months old (66.1 ± 24.4 cm ATL). In the Avilon Zoo in Manila, samples were taken from 29 Philippine crocodiles (hatchlings, n = 12, ca. 8 weeks, 29.8 ± 0.8 cm ATL, juveniles n = 14, ca. 14–43 months old, 62.5 ± 30.8 cm ATL, and adults, n = 3, 15–25 years of age, 216.0 ± 14.5 cm ATL). Also, 2 adult crocodiles (between 15–20 years of age, 183.8 ± 18.0 cm ATL) from the Ilagan Mini Zoo in the Fuyot Spring National Park, Isabela were sampled.

2.2 Housing and diet Thirty crocodiles were housed individually in partly open steel drums, with fresh water and a land area, for 8 weeks. Fifteen crocodiles (50%) received a plastic hiding board of 28 cm (width) × 32 cm (length) × 12 cm (height), while fifteen did not receive a hiding board. In both groups, with and without hiding board, 3 sub-groups of 5 crocodiles each received three different diets three times a week (35 g each time); group 1 meat (n = 2 × 5), group 2 shrimps (n = 2 × 5) and group 3 a mix of meat and shrimps (n = 2 × 5). Air temperature in the drums was 29.5 °C ± 5.5 °C (mean ± SD), water temperature was 24.8 °C ± 2.5 °C, and humidity was 84.75 % ± 13.25 %.

2.3 Health status A heath check was done whenever saliva samples were taken. The total body length, snout-vent length (SVL), tail length, head length, neck and tail circumference and weight were measured to monitor growth. Furthermore, the crocodiles' joints, skin, mouth and eyes were studied for any clinical signs of disease or injury.

2.4 Enclosure use The location of the reared crocodiles in the drums was recorded (in water, on land or under hiding board) 3 times a day, at 07:00 h, 13:00 h and 21:00 h for 14 days within 2 months.

2.5 General hemolysis-hemagglutination assay Assays were carried out at the University of the Philippines

in Los Baños and done in 96-well (eight rows by 12 columns) round (U) bottom assay plates (adapted from Wegmann and Smithies, 1966; Matson *et al.*, 2005) in duplicate. Twenty-five microliters (µl) of PBS was pipetted into columns 1–12 of every row. From every sample 25 µl was pipetted, sample 1 in column A, well 1, sample 2 in column A, well 2 and so on following a two-fold serial dilution. Next, 25 µl of column A, well 1 was pippeted to column B, well 1 etc. This was also done for the columns 2–12. Twenty-five µl of 1% sheep blood was pipetted in every well of the plate. The plates were gently tapped against a solid edge by hand for 60 sec, and left for incubation on the table for 90 min.

2.6 Statistical analyses Statistical analysis of experimental data and NAb titres were performed using an independent *T*-test in IBM SPSS Statistics 19. The statistical analyses of the NAb titre data of all samples were done using a Pearson Correlation in SAS 9.2 (SAS Institute, Cary, NC, USA).

3. Results

3.1 Health status Five different diseases were found, and two of them could not be identified (Table 1). The two crocodiles in the Ilagan Mini Zoo did not show clinical signs of disease. Hiding board and diet did not affect growth rate.

3.2 Enclosure use Crocodiles that were housed without a hiding board were observed more often in water than the crocodiles with a hiding board (80.7% vs. 26.4% of the observations, morning and midday $P < 0.0001$ and evening $P < 0.01$; Figure 1). Crocodiles used the hiding boards in 69.7% of the observations (3.1% on top of the hiding board), with the highest incidence of board use in the morning and midday (89.1% and 83.9%, respectively).

3.3 NAb titres A positive correlation was found between NAb titres and body size (including head length, SVL, tail length and total length, $r = 0.44$, $P < 0.0001$; Figure 2), weight (including neck and tail circumference, $r = 0.42$, $P < 0.01$) and age ($r = 0.39$, $P < 0.0001$). Crocodiles of 4–5 months old at MPCRS had a higher titre than those of the same age in the Avilon Zoo ($P < 0.005$). No difference was found between the crocodiles of other ages. Also, no effect of housing or diet on the NAb titres and no interactions were found.

Table 1 Diseases and affected areas found in the Philippine crocodiles at MPCRS (n = 66) checked 3 times and in the Avilon Zoo (n = 30) checked once.

		Location	
Disease	Affected area	MPCRS	Avilon Zoo
Chronic stress dermatitis	Around nostrils and eyes	5	0
Osteomalacia	Mouth	1	2
White faeces	Intestine	5	0
Fungal disease	Groin and tail	2	0
Deformities	Jaw and tail	1	1

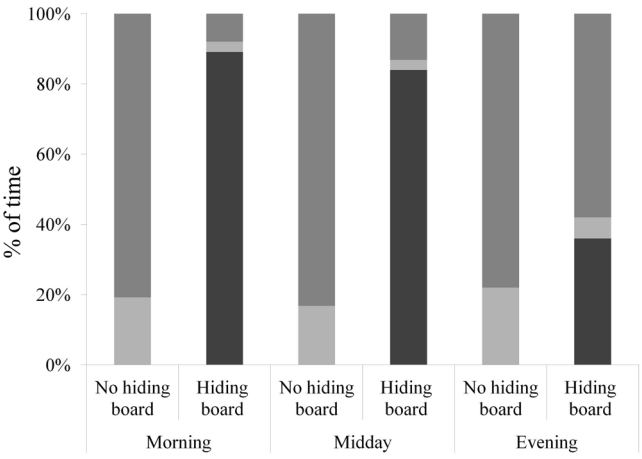

Figure 1 Enclosure use of crocodiles housed with (n = 15) and without hiding board (n = 15) in 3 possible locations: land (light grey), water (grey) and hiding (black; when hiding board provided) in the morning, at midday and in the evening.

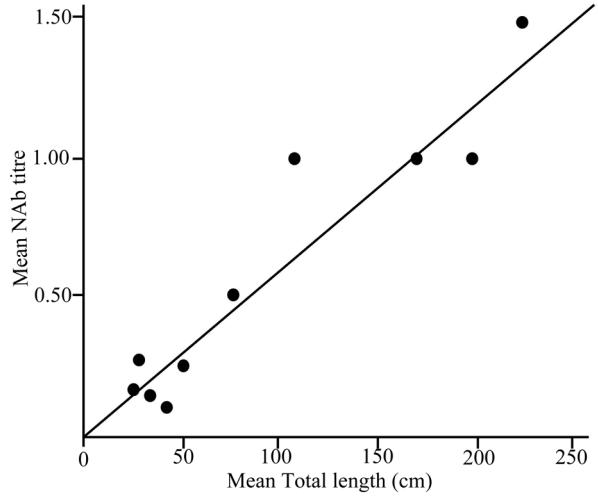

Figure 2 Mean NAb titre in saliva in relation to the mean total body length of the crocodiles (n = 97).

4. Discussion

In the current study, five diseases were found, where only three could be identified. Overcrowding of crocodiles probably caused chronic stress dermatitis in two juveniles at MPCRS. The boneless diet provided at MPCRS and in the Avilon Zoo could have caused calcium shortage and resulted in an early stage of osteomalacia in three

hatchlings. It was recorded as early state osteomalacia, because the hatchlings had a partly flexible jaw and they were still able to move on land, which is not possible when the osteomalacia becomes more serious (Huchzermeyer, 2003). Two hatchlings at MPCRS had fungal dermatitis where the skin was shed, probably caused by lack of hygiene in the drums, but no specific fungus could be identified. Five crocodiles had white faeces, often caused by uric acid crystals, but even experts were not able to identify the cause (pers. comm. Huchzermeyer, 2012).

The current study aimed to define an optimal rearing strategy of Philippine crocodile hatchlings. In our experiment, crocodiles without a hiding board spent most time in water, while crocodiles with a hiding board spent most time under the hiding board as a shelter. This could be due to that hatchlings are afraid of any movement overhead, even indoors (Huchzermeyer, 2003). Overhead movement-stress can be minimized with a hiding board, resulting in a better working immune system. While we hypothesized that crocodiles with hiding boards had a higher growth rate and higher level of NAbs, the present data showed that hiding boards did not affect growth rate and NAb level, probably because the crocodiles at MPCRS still perceived other stresses from outside noise, handling, cleaning, visitors and low water temperature (lower than optimal temperature at all times, 32°C; Huchzermeyer, 2003).

We expected that a mixed diet would increase the NAb levels and decrease diseases prevalence due to its influence on intestinal mircobiota (Haghighi *et al.*, 2006). However, changes were not evident within the 3 months of the experiment, possibly due to other rearing-related stress issues.

A positive correlation was found between NAb titres and body size, weight and age of Philippine crocodiles. This could be due to the exposure to less exogenous stimuli in young animals compared to older animals, similar to a recent study of Nabs in pythons (Ujvari and Madsen, 2011). However, in other species such as poultry (Berghof *et al.*, 2010) and mammals (Cheng and Chamley, 2008) it is shown that NAb titres not only depend on exogenous stimuli, but also are related with auto-antigens. Hatchlings captured from the wild had higher NAb titres compared to those incubated in the Avilon Zoo of the same age. Wild hatchlings likely encountered more exogenous stimuli (Prokesova *et al.*, 1996), including intestinal mircobiota (Haghighi *et al.*, 2006), causing antigen nonspecific activation of B cells via toll-like receptor triggering (Lanzavecchia and Sallusto, 2007). This may have resulted in enhanced continuous polyclonal production that shaped the repertoire and enhanced the level of NAbs (Prokesova *et al.*, 1996). Hatchlings from the zoo were born in an incubator with little stimuli resulting in lower NAb levels. The difference diminished at older age. In both locations, older crocodiles were housed outside in semi-natural ponds with many exogenous stimuli, resulting in similar NAb levels in crocodiles of MPCRS and Avilon zoo.

Whilst hiding boards and diet did not affect growth rate and NAb titres in the current study, literature does suggest its importance for stress reduction and disease occurrence. So, we do recommend the provision of hiding places and a diverse diet in crocodile husbandry. Furthermore, we recommend to provide exogenous stimuli for juvenile crocodiles that are reared to be released into the wild, for example, by rearing them in semi-natural ponds for a few months prior to the release. These additions could all benefit the recovery of the critically endangered Philippine crocodile.

Acknowledgements We would like to thank F. Huchzermeyer for his help in identifying the diseases and E. Lastica for helping with the laboratory work. Furthermore, we want to thank the staff of the Avilon Zoo and Ilagan Mini Zoo for allowing and assisting the collection of the saliva samples. Special thanks go to the staff of the Mabuwaya Foundation for all their help in data collection. This study was facilitated by Isabela State University and the Mabuwaya Foundation, and the experiment was approved by the Animal Care and Use Committee of Wageningen University and Research Centre. The Philippine crocodile head-start program of the Mabuwaya Foundation is implemented under a Gratuitous permit by the Department of Environment and Natural Resources.

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