Report of Student Research Assistance Scheme

Project title: "Immunological investment of broad-snouted caiman (*Caiman latirostris*) under situations of stress"

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Introduction

Caiman latirostri, is one of the 2 species of crocodilians that inhabit the Argentine territory, reaching its southernmost distribution to the Santa Fe province. Prefers densely vegetated environments with an abundance of food. Frequently caimans must face a changing environment, such as periods of low hydrometric levels, where densities are detected in scarce places with water, causing overcrowding, food restriction, etc.

Consequently, these situations are considered as stressors and can trigger disputes between congeners causing serious injuries and even the loss of a member. In spite of the severity of these wounds and the high concentration of pathogenic microorganisms that may appear in the places where they live, no signs of infection are commonly detected. Given the importance of defense mechanisms, any factor that implies some alteration in their functioning could have potential implications for both conservation and sustainable development programs. In order to characterize the behavior of the immune system of broad-snouted caiman (*Caiman latirostris*) under stressful situations, we represent what would happen in the wild, in presence of three potential environmental stressors.

Materials and Method

An experiment was carried out using *C. latirostris* specimens from nests harvested in Natural Reserve "El Fisco", an area free of farming and urban activities (30°11'26"S, 61°0'27"O; San Cristóbal, Santa Fe, Argentina; Provincial Law 12.930/2008) and artificially incubated at the Yacaré Project site.

This work had two phases, with a month of duration each other. The first one consists in an experiment with 60 individuals of 3 months old, coming from 4 nests and randomly distributed in 3 treatments (in duplicate): water restriction (WR), food restriction (FR), heat stress (TMP) and control. During this period I assess the effects of this stressor over corticosterone levels and immunological parameters (total and differential account of leukocytes, natural antibody and complement system). In the second phase, the stressors were removed to observed changes in

immunological parameters and in corticosterone levels over time (10, 20 and 30 days). At all phases the animals were weighed, measured and body condition was calculated.



Fig 1. Egg collection of *C. latirostris.*



Fig 2. *C. latirostris* under temperature treatment.

Blood was extracted from the spinal vein for the determinations:



Corticosterone Assay : Corticosterone concentrations in plasma were measured by radioimmunoassay (RIA) after extraction with diethyl ether as previously described (Jahn *et al*., 1995) and modified for *C. latirostris* by Parachú Marcó *et al*. (2014). Samples were run in duplicate.

Determination of complement hemolysis test system (CS): To characterize the *C. latirostris* SC, the SRBC (Sheep red blood cells) were obtained from heparinized whole blood drawn from merino sheep (*Ovis aries*).

Sheep blood was centrifuged at 450 g for 20 min and the supernatant was discarded, performing repeated washings with a phosphate buffered saline solution (PBS, pH 7.4) until the supernatant was clarified. After the final wash, a solution of 2% (v / v) SBRC in PBS was prepared.
Yacaré plasma was incubated with SRBC for 30 minutes, then the mixed was centrifuged at 2500 x. Subsequently, 300 ul of supernatant were taken and transferred to a microplate for measuring the optical density in a microplate reader at 540 nm (Multiskan RC Labsystem, Helsinki, Finland). The result obtained was considered as the maximum hemolysis (MH).

Determination of natural antibodies (Nab): Determination of NAbs titres (dilution factor -1) was carried out in triplicate, using a hemagglutination assay described by Matson et al. (2005), with modifications. The assay is carried out in 96-well bottom microplates.
Twenty-five µl of RRBC and 25 µl of 0.01 M phosphate buffered saline (PBS) were added in 12 consecutive columns. Caiman plasma was added only in the columns 1. Using a dilutor (fixed volume) or pipette, 25 µl of the contents in the column 1 were added to the contents of the column 2 wells through column 11(serial dilution 1:2) and 25 µl of the column 11 were discarded. The column number 12 was negative control (PBS + RRBC). Each plate is then sealed with Parafilm and protected of the light during 90 min.

Total leukocyte count (TWBC): An aliquot of blood was taken and diluted with 0.6% NaCl (Lewis et al., 2008), in a 1: 200 dilution and added in a Neubauer chamber. The samples were observed with an optical microscope (450x) using a Newbahuer chamber. Results were expressed as number of cells per cubic millimeter of blood.

Differential leukocyte count (DWLC): two smears were made per animal. Blood was fixed with ethanol for 10 min and then be stained with May Grunwald solution - Giemsa. Then they were observed under an optical microscope (1000x). The amount of the subtypes of white blood cells (heterophils, basophils, eosinophils, lymphocytes and monocytes) were expressed each 100 cells analyzed and expressed as percentage.

Result

First phase

Stress treatments had dissimilar effects on the weight, SVL and body condition. Those who were exposed to temperature stress weighed on average 50% more than controls (p<0.0001), whereas those in the food deprivation group 36% less (p<0.0001). Individuals from WR and FR were significant shorter than controls (0.5 cm and 1.1 cm on average, respectively), whereas those

exposed to higher temperatures had on average 1.2 cm more than controls. Regarding body condition, caiman under FR treatment were on average 26.5% lower than controls (p<0.0001). In turn, individuals in TMP groups had body condition indices 36.5% higher (p<0.0001) than controls.
Plasma corticosterone levels (ng/ml) at the end of the experiments were not found to be increased by any of the stress treatments. Individuals from WR and FR were not different from controls (p=0.6577 and p=0.5248 respectively). Conversely, caimans exposed to high temperature showed 49.5 % lower corticosterone levels than controls (p<0.0029).
Counts of white blood cells, all combined or by leukocyte type, were in all cases not statistically different between treatments. Also, no significant differences were found in activity of complement system (MH%-percentage of maximum hemolysis). Individuals from TMP and FR groups showed significantly higher NAbs titers than controls (p=0.00177 and p= 0.04419, Fig. 3).
Second phase
After the treatments were removed, caiman that had been under food restriction (FR) continued to have the same difference in weight, SVL and body condition compared with controls. Similarly it happened to those who had been exposed to high temperatures.
We found only difference, at 10 days of removed treatments, in the corticosterone levels (p= 0.0542) of those individuals who had been exposed to food restriction compared to controls. At 20 days after the treatments were removed, difference was observed in the total white blood cell count (p = 0.00229) and in the lymphocyte count (p = 0.00951) of the individuals who had been under treatment without food compared with controls. This difference was maintained at 30 days after the treatments were removed. In addition, at this time we found difference in the % of max haemolysis (p = 0.0221) in individuals who had been under FR.

Hormone analysis is a precise used tool for monitoring responses to stressors, non-invasive methods have gained popularity over the past 30 years as a more practical approach for assessing adrenocortical activity in wildlife species (Ganswindt et al., 2014). Also, the immune system is an excellent indicator of the health of an organism (Burns et al., 1996). However it is widely accepted that prolonged (chronic) stress decreases immune function through mechanisms involving the hypothalamic-pituitary-adrenal (HPA) axis and glucocorticoids, leaving individuals more susceptible to infection (Sapolky, 2000).
In the first phase of our study, the only negative effects were detected in growth parameters in animals that were deprived of food. The effect of high temperature appeared to be beneficial because individuals exposed to heat treatment (TMP) grew more, had better body condition, higher levels of Nab and corticosterone levels were significantly lower than those of caiman in all other

groups. This suggest that *C. latirostris* is a species that tolerates environmental stressors to which it can be exposed and also are benefited by the high temperatures, allowing them to invest in humoral immunity. However, in the second phase of our experiment, we observed that food restriction treatment caused a decrease in immune function showed a long-term negative effects.

- Actually this Manuscript is under review in "Journal of Zoology"

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