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Corticosterone levels and immunological indices in stressed juvenile broad-snouted caimans

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Abstract

The effect of stress in wild vertebrates has been little investigated, especially in crocodilians. In the wild, crocodilians face numerous stressors such as climatic factors, toxicant exposure, and infection. Exposure to stressors may affect the physiological processes of crocodilians, with consequences on its fitness and survival. We experimentally investigated the effect of a 4-week exposure to environmental challenges (food restriction, water restriction and high temperature) on growth, immunological investment and corticosterone levels of juvenile *Caiman latirostris*. White blood cells counts, natural antibody (NAb) levels and complement system activity were evaluated to characterize the influence of those treatments on the immune system. We found deprivation of food resulted in reduction in growth and body condition, whereas high temperature had a beneficial effect on the variables measured. Individuals exposed to heat treatment (37 ± 1°C) grew more, and obtained better body condition, higher NAb levels, and significantly lower corticosterone levels relative to caimans in all other groups. Food restricted caimans also illustrated higher NAb levels. Our findings suggest that *C. latirostris* juveniles are able to tolerate environmental stressors and they are even favored by high temperatures.

Introduction

Stress is defined as the physiological and behavioral response to unpredictable and/or uncontrollable demands that exceed the regulatory capacity of an organism (Koolhaas *et al.*, 2011). In vertebrates, the stress response is modulated by glucocorticoids (GCs) (Moore & Jessop, 2003; Meylan, Haussy & Voituron, 2010), which activate a cascade of physiological and behavioral responses. The production of GCs is regulated by the hypothalamic–pituitary–adrenal (HPA) axis in mammals and birds, and by the hypothalamic pituitary–interrenal (HPI) axis in fish, amphibians and reptiles (Sopinka *et al.*, 2015). Some of the physiological effects triggered by GCs are the mobilization of energy from adipose and hepatic tissues, the shutdown of digestive processes, and immunosuppression (Meylan *et al.*, 2010).

Although it is generally accepted that immunity is down-regulated during chronic stress responses, data from rodents have shown the opposite. Capybaras (*Hydrochoerus hydrochaeris*) exposed to prolonged stress (twelve weeks of food limitation or physical restriction) showed elevated eosinophil counts, and food restricted animals had higher levels of natural antibodies (Eberhardt *et al.*, 2013). This demonstrates that there is no general pattern regarding the effect of chronic stress on the immune function, and suggests strongly that this effect may depend on the natural history of a given species (Beldomenico & Begon, 2015).

Crocodilians are an ancient group of archosaurs, composed of three distinct familial lineages: Alligatoridae, Crocodylidae and Gavialidae. They are opportunistic, top trophic carnivores inhabiting semi-aquatic environments (Finger & Gogal, 2013). They also have complex behaviors that involve violent interactions, which result in serious wounds, including the loss of entire limbs. However, they do not show signs of infection, despite that these animals continually live in contact with potentially pathogenic agents in their habitat (Siroski *et al.*, 2009; Siroski, 2011). Several studies have indicated that crocodilians exhibit potent immune capabilities that allow them to overcome such conditions. The antimicrobial activity of crocodilian blood was described by Merchant *et al.* (2003, 2004); Merchant & Britton (2006) in serum of *Alligator mississippiensis*; and Siroski *et al.* (2009) reported that antibacterial activity in *Caiman latirostris* serum is higher than that of hen and human plasma.

Whether the antibacterial immunocompetence of crocodilians might be impaired under certain conditions, such as exposure to stressors, is not well known. The information related to the effect of stressors on crocodilian physiology is limited to a few studies that examined the impact of capture method, crowding and other forms of stress on growth (Elsey *et al.*, 1990; Morici, Elsey & Lance, 1997), hematological values (Turton *et al.*, 1997) and immunity (Finger *et al.*, 2015). Herein, we examine the effect of a 4-week exposure to the most commonly faced environmental stressors (i.e. water restriction, food restriction, and elevated temperature; Moore & Jessop, 2003; Johnstone, Reina & Lill, 2012) on growth, immunological investment and other physiological parameters of juvenile *C. latirostris*.

Materials and methods

Animals and husbandry

Caiman latirostris eggs were harvested from the 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) during 2015 and 2016 years. After collection, eggs were incubated at 31 ± 1°C at facilities of Proyecto Yacaré (PY), in an artificial incubator placed at the Laboratorio de Zoología Aplicada: Anexo Vertebrados (FHUC - UNL/MMA, Santa Fe). Immediately after hatching, we selected 192 juvenile caimans from eight clutches, cut a scute for identification, and housed the individuals in 16 pens (40.4 cm in width and 78.5 cm in length). Each pen had both dry and wet areas, and fed minced chicken head *ad libitum* to the animals. We changed water every 2 days alternating with feeding days. Pen temperature remained at 30 ± 1°C and monitored with Hobo™ data loggers (Onset Computer Corp., Pocasset, MA, USA).

Experimental design

Before the beginning of the experiment, we weighed, measured (TL = Total Length, SVL = Snout Vent Length) and randomly distributed animals into 4 treatment groups in duplicate, 24 animals per treatment, distributing clutches homogeneously. Commencement of stress treatments began at approximately 60 days of age. Treatments of groups are as follows: (1) control (CTL): animals kept in the same conditions that preceded experimental treatments; (2) water restriction (WR): water level reduced to 50% in pen. This treatment attempted to simulate a moderate to severe drought (Lance *et al.*, 2010); (3) food restriction (FR): animals did not receive food during the experiment (Cote *et al.*, 2010); and (4) high temperature (TMP): air temperature was maintained at 37 ± 1°C for 8 h each day. We selected this temperature based on global warming predictions (Killen *et al.*, 2013). After exposure to these stressors for 1 month, we weighed, measured and calculated body condition (Bc = weight/SVL). The exposure time was selected because it is approximately the length of time we expect the stressors investigated to be present in nature. For instance, at the study area, average temperatures over 30°C are recorded mostly in January, being less frequent in preceding and subsequent month (http://siga2.inta.gov.ar). In addition, longer exposures might result in the development of tolerance to these type of stressors (Romero, 2004), and consequently the animal no longer responds in the same robust manner to chronic stressors. Previous experimental studies of adult rats demonstrated that after 2 weeks of handling the GC response to handling was significantly reduced compared to their initial response (Dobrakovová *et al.*, 1993).

Samples

This study was evaluated and approved by the ethics and safety committee of the Facultad de Ciencias Veterinarias of the Universidad Nacional del Litoral (Santa Fe, Argentina) (N°301/15) and is in accordance with the 'Guide for the Care and Use of.

Agricultural Animals in Agricultural Research and Teaching' https://www.aaalac.org/about/Ag_Guide_3rd_ed.pdf).

We collected blood samples at the end of the experiment from the spinal vein with heparinized syringe to analyze total white blood cells (TWBC) counts and estimation of the proportion of leukocytes. We then centrifuged the blood for further analyses in order to separate the plasma, which was stored at −80°C until assayed.

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.

Immunological indexes

Total white blood cells (WBC) counts

We used a Neubauer chamber to determine the total number of leukocytes after diluting an aliquot of whole blood with a solution of 0.6% NaCl at ratio of 1:200. Analysis occurred using an optical microscope at 400× and expressing results as total cells μL^{-1} blood (Lewis, Bain & Bates, 2008).

Differential white blood cells (WBC) counts

Calculation of differential leukocyte count included making 2 smears per animal fixed with ethanol, and then stained with May Grunwald–Giemsa solution and observed under an optical microscope at 1000×. We examined smears and counted each type of leukocyte (heterophil, basophil, eosinophil, lymphocyte and monocyte) to establish a percentage. These values and the TWBC count were then used to estimate the concentration of each leukocyte type in blood (cells μL^{-1}). We coded each preparation to achieve maximum objectivity in the analysis.

Natural Antibody Assay (NAbs)

To evaluate constitutive immunity, we measured the levels of natural antibodies (NAbs) in blood using the hemagglutination

method, which is based in the antigen-antibody interaction between NAbs and rabbit red blood cells (RRBC). Lysis reflects the interaction of complement and NAbs, whereas agglutination results from NAbs only (Matson, Ricklefs & Klasing, 2005). We washed rabbit blood several times with phosphate-buffered saline (PBS, pH 7.4) until the supernatant was clear, and then prepared a 1% RRBC (v/v) solution. The plasma was serially diluted in PBS in U-shaped microtiter plates followed by an equal volume of RRBC in NaCl (0.15 M) (RRBC 1%). We covered plates with parafilm after homogenization and incubated plates at an ambient temperature (25 ± 2°C) for 2 h. We then visually determined agglutination. Titers were expressed as the log 2-transformed reciprocal of the highest dilution of plasma showing hemagglutination.

Complement system activity

According to Siroski *et al.* (2010), the assay to detect activity of complement system called SRBC (Sheep red blood cells) hemolysis assay is based on the hemolytic disruption of SRBCs by means of serum immunological proteins. Collection of heparinized whole blood from the jugular vein of Merino sheep (*Ovis aries*) provided fresh SRBCs needed for our experiment. We washed sheep blood several times with phosphate-buffered saline (PBS, pH 7.4) until the supernatant was clear, and then prepared a 2% SRBC (v/v) solution.

Tests occurred at laboratory ambient temperature (25 ± 2°C) by placing 0.5 mL of plasma from each animal together with 0.5 mL SRBC solution at 2% (v/v). After 30 min incubation, the mixture was centrifuged and 300 μL of supernatant was used for analysis on microplate reader.

We obtained a positive control for hemolysis by 1% SRBCs solution and 1% (v/v) Triton X-100. Complete hemolysis occurred after injecting and ejecting the mixture several times through a tuberculin syringe. We measured optical density of the supernatant in a microplate reader at 540 nm, and calculated the maximum hemolysis. Results were expressed as the percentage of maximum hemolysis (MH%; mean ± SE).

Statistical analyses

We conducted baseline comparisons before treatments to confirm that groups did not differ in terms of weight, length (TL and SVL) and body condition. We set α at 0.1, and comparisons were made using analysis of variance (ANOVA).

The main set of analyses was conducted with Linear Mixed Models (LMM) and Generalized Linear Mixed Models (GLMM), using the lme4, languageR and lmerTest package of the statistical software R (R Foundation for Statistical Computing, http://www.r-project.org). We added the random intercept 'Pen ID' and 'year' to take into account the lack of independence of observations from the same pen. We used a GLMM with a negative binomial response to analyze natural antibody titers. For the remaining parameters, LMMs were used. The independent variables were 'treatment' (4 levels) and 'clutch'; the latter to account for the 'nest' effect.

Results

Baseline comparisons showed that experimental groups did not significantly differ in terms of weight ($P = 0.160$), length ($P = 0.464$) and body condition ($P = 0.473$) before treatments were applied. The descriptive statistics of all parameters measured at the end of the experiment are shown in Table S1.

Growth

Stress treatments had dissimilar effects on the weight, SVL and body condition (weight/SVL) (Fig. 1; Table S2). While individuals in the WR group appeared to weigh less than controls, this difference did not statistically differ ($P = 0.6400$). Animals exposed to temperature stress weighed on average 50% more than controls ($P < 0.0001$), whereas those in the food deprivation group weighed 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. Body condition of caimans from WR did not differ significantly compared to controls ($P = 0.8244$), yet the body condition of the FR treatment animals was 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.

Corticosterone

We found that stress treatments did not increase plasma corticosterone levels (ng mL^{-1} by the end of the experiments (Fig. 2). Individuals from WR and FR did not differ from controls ($P = 0.6577$ and $P = 0.5248$, respectively). Conversely, caimans exposed to high temperature showed 49.5% lower corticosterone levels than controls ($P < 0.0029$).

Immunological indices

We found no statistical difference among treatments of white blood cells counts, all combined or by leukocyte type (Table S3). Also, we found no significant differences in activity of complement system (MH%-percentage of maximum hemolysis). In contrast, the effect of treatments on Nabs differed significantly (Table S4, Fig. 3). Individuals from TMP and FR groups showed significantly higher NAbs titers than controls ($P = 0.00177$ and $P = 0.04419$, respectively). Natural antibodies levels did not differ statistically comparing WR and controls (Table S5).

We found no significant difference in the heterophil/lymphocyte ratio (H/L ratio) across stress treatments (WR = 0.5504, FR = 0.9547and TMP = 0.3960).

Discussion

Glucocorticoids have been measured as an indication of stress response in a wide range of taxa, fish (e.g. Fast *et al.*, 2008; Morandini *et al.*, 2014), birds (e.g. Martin *et al.*, 2005; Liebl

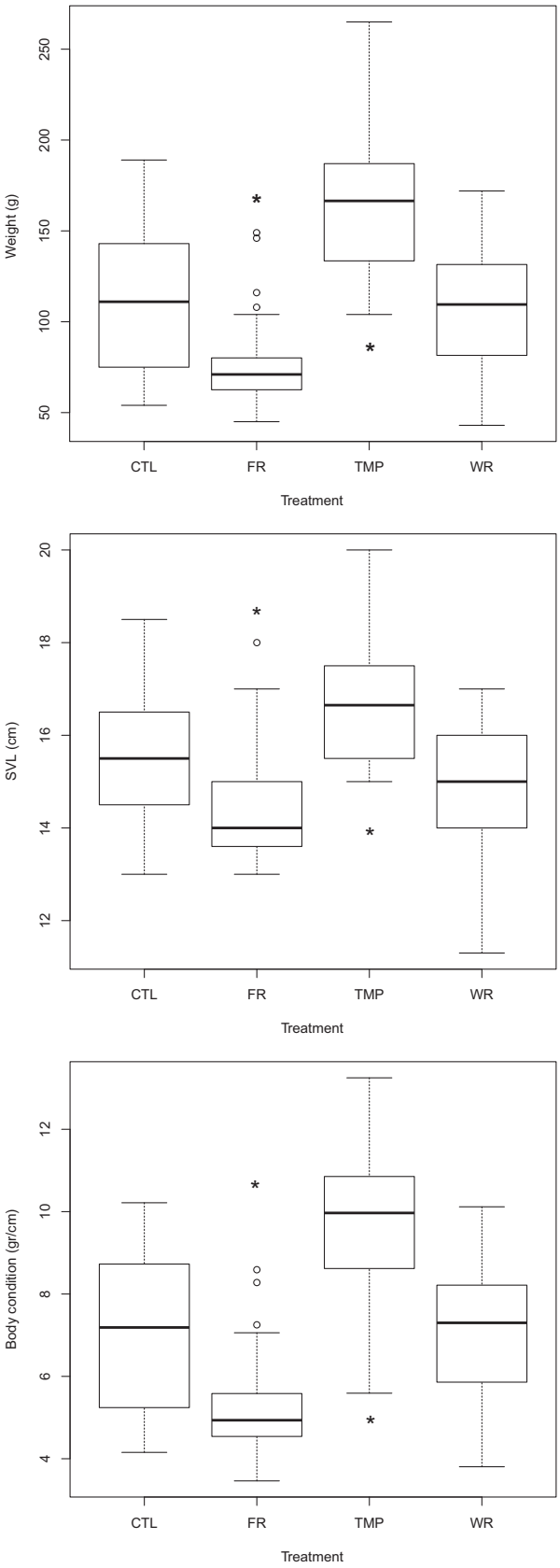

Figure 1 The effect of treatments on growth. Boxplots showing the effect of three different treatments FR (food restriction), TMP (temperature), WR (water restriction) and CTL (control) on weight (a), SVL (b) and body condition (c). Boxplots represent the median (bold bar), 25–75% quartiles (box), 10–90% quartiles (whiskers) and outliers (points). *Indicates significantly difference.

& Martin, 2013), reptiles (e.g. Cote *et al.*, 2010; Meylan *et al.*, 2010) and rodents (Bartolomucci, 2007; Cuervo, 2017). These studies have assessed different types of stressors (e.g. natural or anthropogenic), finding diverse effects. For example, in reptiles, Cote *et al.* (2010) reported that common lizards (*Lacerta vivipara*) under food restriction had high levels of corticosterone, but this did not alter their normal behavior. Extreme temperature, a stressor of special interest for ectotherms, was evaluated by Dupoué *et al.* (2013) in constricting snakes (*Antaresia childreni*), finding that pythons exposed to cold temperature (17°C) had higher corticosterone concentrations compared to those exposed to warm temperature (35°C). The corticosterone values found in this work are within the ranges reported in the studies cited above by Turton *et al.* (1997) (2,2 to 140,5 ng mL^{-1}) in *C. porosus* and Morici *et al.* (1997) in *A. mississippiensis* (3.8 to 42.8 ng mL^{-1}). However, average values were significantly higher than those described in Finger *et al.* (2015) (1.8 ng mL^{-1}) and Parachú Marcó *et al.* (2015) for *C. latirostris* embryos (0.01 ng mL^{-1} to 7 ng mL^{-1}).

Our current knowledge on the effect of stress on crocodilian physiology is limited to only four experimental studies that evaluated temperature, farming practices, crowding and corticosterone implants. A study conducted by Turton *et al.* (1997)

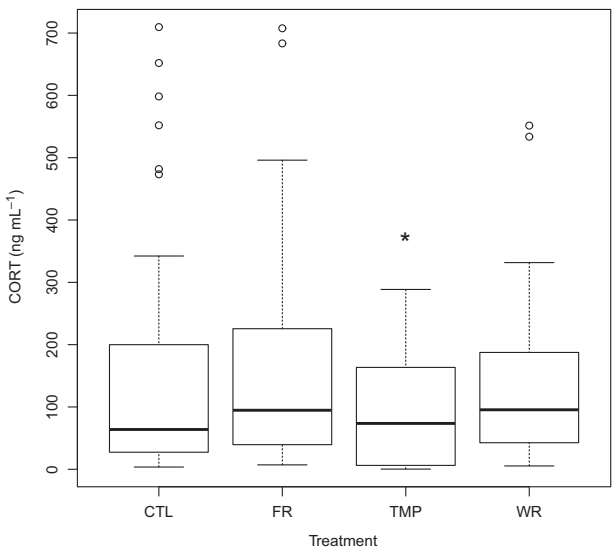

Figure 2 The effect of treatments on plasma corticosterone levels. Boxplots showing the effect of three different treatments FR (food restriction), TMP (temperature), WR (water restriction) and CTL (control) on plasma corticosterona levels. Boxplots represent the median (bold bar), 25–75% quartiles (box), 10–90% quartiles (whiskers) and outliers (points). *Indicates significantly difference.

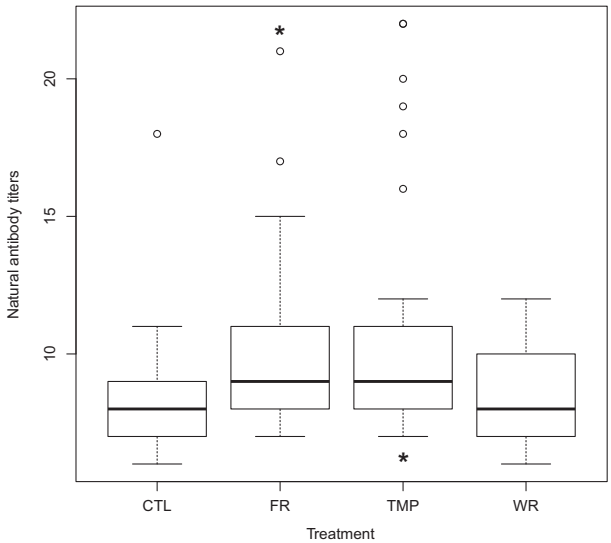

Figure 3 The effect of treatments on Natural Antibody titers. Boxplots showing the effect of three different treatments FR (food restriction), TMP (temperature), WR (water restriction) and CTL (control) on natural antibody titers, expressed as the last serum or plasma dilution showing clear evidence of agglutination. Boxplots represent the median (bold bar), 25–75% quartiles (box), 10–90% quantiles (whiskers) and outliers (points). *Indicates significantly difference.

in *Crocodylus porosus* reported that animals exposed to low water temperature (28°C) had decreased hematological values, whereas those exposed to high water temperature (36°C) showed increased corticosterone levels and reduced growth, compared to controls (32°C). A study in *A. mississippiensis* produced evidence of the stressing effect of crowding, as alligators maintained at the lowest stocking density (0.18 m^2/Alligator) grew significantly faster than those maintained at high stocking densities (0.12 m^2/Alligator) (Elsey *et al.*, 1990). Further, stocking densities directly correlated with corticosterone levels. Similarly, Morici *et al.* (1997) carried out an experiment injecting corticosterone in alligators, and found those animals that received corticosterone experienced a reduction in growth and had decreased percentages of lymphocytes, eosinophils, and basophils. In contrast, Finger *et al.* (2015) showed that corticosterone injection in hatchlings of *C. porosus* failed to affect growth, innate immunity and cellular immunity.

In our study, deprivation of food caused the only negative effects. Snout vent length, body mass and body condition were significantly lower in caimans in 'FR' groups compared with other groups. This effect of nutritional stress on growth parameters is expected and has been documented in several taxa (Kitaysky *et al.*, 2003; Cote *et al.*, 2010). Nonetheless, the strong effect in growth and body condition is in contrast with the lack of significant difference in corticosterone levels between in FR caimans and controls, which suggests that this failure to find a statistically significant difference may be due to lack of statistical power. This could be attributed to the large variances observed in corticosterone levels.

High temperatures are known to be stressful in ectotherms and may therefore elicit an activation of the HPA axis (Lourdais, Heulin & Denardo, 2008). However, in our experiment the effect of high temperature appeared to be beneficial in several variables measured. Individuals exposed to heat treatment (37 ± 1°C) grew more and had better body condition, and higher levels of NAb. In addition, we found no evidence of a stress response in those housed at 37°C, in addition to significantly lower corticosterone levels than those of caimans in all other groups. Similarly, pythons exposed to acute heat stress (35°C for 48 h), unexpectedly presented lower corticosterone levels compared to baseline corticosterone levels before heat exposure. In laying hens, elevated temperatures (37°C) resulted in the opposite: anorexia and weight loss (Mashaly *et al.*, 2004). It should be taken into account that the environmental temperature is important for food intake, behavior, and digestive processes in crocodiles. In *A. mississipiensis* and *C. acutus*, the optimum temperature range for feeding is considered to be between 25 and 35°C (Lang, 1979; Turton *et al.*, 1997). The optimum temperature range for *C. latirostris* is between 23°C and 35°C (Basetti *et al.*, 2014), but the results reported suggest that caimans perform better at higher temperatures. Other authors reported enhanced growth in *C. latirostris* at high temperatures. Parachú Marcó *et al.* (2010) found that the rearing temperature had a profound effect on hatchling growth. Animals kept at 33°C had grown more than those kept at 29°C. Appetite may ultimately depend on the availability of warm temperatures to digest and assimilate food (Lang, 1987). Digestion is directly affected by temperature, increasing temperatures result in more appetite, elevated gastric contraction frequency and amplitude, and peptic activity. An explanation for the lower corticosterone levels in caimans exposed to 37°C might be that the sensitivity of the adrenocortical function may be dependent of the body condition of crocodilians (here, animals under heat stress had better body condition). This has been suggested by Jessop *et al.* (2003), who found that Australian freshwater crocodiles (*Crocodylus johnstoni*) under capture stress had differential corticosterone levels depending on their body condition: the stress response was lower the better the body condition.

Surface water is also an important resource in the life history of crocodilians because it is involved in many physiological processes. Evaporation increases when temperature increases, suggesting a potential interaction between thermoregulation and water balance in ectotherms (Lorenzon *et al.*, 1999). Lizards and snakes subjected to total water restriction for 1 month experienced significant body weight loss and increases in plasma corticosterone (Lorenzon *et al.*, 1999; Dupoué *et al.*, 2013). We expected corticosterone levels to be higher in juvenile of *C. latirostris* under water restriction treatment as a result of hydric stress, but the difference observed did not statistically differ. This might result from poor statistical power as argued above for the food restriction treatment, but we observed no effect in the physiological parameters evaluated, likely illustrating that 50% water restriction failed to trigger a stress response. Perhaps more severe restrictions would have resulted in measurable stress.

Previous studies performed on crocodilians and other reptiles showed an association between high corticosterone levels and depression of the immune function (Morici *et al.*, 1997; Berger

et al., 2005; Martin, 2009). We could not confirm such findings. Indices of the immune function varied significantly across individuals, yet we did not observe any evidence of reduced immunological defenses, not even in the treatment group where we observed the effects of food restriction. Moreover, heat stress and food restriction caused an increase in the levels of natural antibodies.

Natural antibodies (NAbs) are part of the constitutive (innate) immune system and are unique among immunoglobulins molecules because their presence does not require previous exposure to a particular antigen (Martin, 2009). Natural antibodies react with various affinities to a wide variety of epitopes on macromolecular and particulate antigens including foreign red blood cells (RBCs), bacteria, viruses, toxins and should be less sensitive to short-term variations in environmental conditions, nutritional status, or stress levels (Matson *et al.*, 2005). Interestingly, the FR group was the only group in which fitness of caimans seemed to be affected. A similar effect was observed in a rodent that lives in sympatry with caimans, the capybara. Capybaras exposed to prolonged food restriction grew less and had very poor body condition, but had higher NAb titers than individuals fed *ad libitum* (Eberhardt *et al.*, 2013). Laboratory rats also illustrate poor body condition and higher NAb titers when deprived of food with the addition or combination of social conflict (Cuervo, 2017). This enhancement of the immune response at times when there is lack of resources strongly suggests that in these species chronic nutritional stress induces an enhancement of some compartments of the immune function, and not the opposite. This might be interpreted as a stress-induced prophylaxis. In the life history of some species, chronic limitation of food might be associated with higher infection risk (Beldomenico & Begon, 2015), so enhancing some compartments of the immune system during these periods might prove a good strategy to maximize probabilities of survival.

Regarding increases in NAb levels in individuals kept at higher temperatures, this might be interpreted as a useful immune investment during a critical period. Several authors mentioned wound and injuries in some crocodilians species as a consequence of severe disputes (Siroski *et al.*, 2009; Isberg & Shilton, 2013). This social behavior becomes more conspicuous in breeding seasons, which occur in parallel with the times of higher temperatures, and greater exposure to pathogens where *C. latirostris* lives. The same applies to food restriction: increased competition for limited resources may result in greater incidence of injuries, and therefore increasing NAb levels as a response to food limitation would also be a reasonable strategy.

This is the first study to explore the effect of selected stressors on the fitness of *C. latirostris*. Our findings contribute to our knowledge of the natural history of this species and in crocodilians in general, and suggests that *C. latirostris* is a species that tolerates exposure to environmental stressors, particularly benefiting from high temperatures. Further research is needed on the stress-immune system interaction of caiman and other wild animal species in order to compare the immunological investment within and between species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Resumen.
Table S1. Mean, minimum and maximum values for growth, Bc (Body condition), CORT (Corticosterone), NAb (Natural antibody) and CS (Complement system) measured in *C. latirostris* in each stress treatment. CTL (Control), WR (water restriction), FR (Food restriction), TMP (Temperature).
Table S2. Mean ±SD for total white blood cell (TWBC), and by cell type (in cells/μL and percentages) measured in *C. latiro*stris under four stress treatments. CTL (Control), WR (water restriction), FR (Food restriction), TMP (Temperature), WBC (Total wite blood cell count), H (Heterophil), E (Eosinophil), L (Lymphocyte) and M (Monocyte).
Table S3. Models describing the effect of treatments on somatic parameters. *indicates significantly difference.
Table S4. *P*-values estimated by the model assessing the effect of treatments on WBC.
Table S5. Models describing the effect of treatments on Natural antibody titers. *indicates significantly difference.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.

The effect of stress in wild vertebrates has been little investigated, especially in crocodilians. We experimentally investigated the effect of exposure to environmental challenges (food restriction, water restriction and high temperature) on growth, immunological investment and corticosterone levels of juvenile *Caiman latirostris*. The only negative effect observed was significant reduction in growth and body condition in caimans that were deprived of food. Individuals exposed to heat treatment (37 $\pm$ 1°C) grew more, had better body condition and higher levels of NAb, and corticosterone levels were significantly lower compared to caimans in all other groups.