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ORIGINAL PAPER

Irregularly calcified eggs and eggshells of *Caiman latirostris* (Alligatoridae: Crocodylia)

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Abstract We describe irregularly calcified egg and eggshell morphologies for the first time in nests of the broad-snouted caiman, *Caiman latirostris*. Research is based on detailed descriptions of 270 eggs from a total sample of 46,800 collected between 2005 and 2011 in Santa Fe Province, Argentina, and encompasses animals from both natural habitats and held in captivity. We discuss possible reasons for the occurrence of eggs with different mineralisation patterns in our extensive *C. latirostris* field sample and its conservation significance; the chemistry of egg laying in amniotes is sensitive to environmental contamination which, in turn, has biological implications. Based on our egg sample, we identify two caiman eggshell abnormalities: (1) regularly calcified eggs with either calcitic nodules or superficial wrinkles at one egg end and (2) irregularly calcified eggs with structural gaps that weaken the shell. Some recently laid clutches we examined included eggs with most of the shell broken and detached from the flexible membrane. Most type 1 regularly calcified eggs lost their initial calcified nodules during incubation, suggesting that these deposits do not affect embryo survival rates. In contrast, irregularly calcified caiman eggs have a mean hatching success rate of 8.9 % (range 0–38 %) across our sample compared to a mean normal success of 75 %. Most irregularly calcified caiman eggs probably die because of infections caused by fungi and bacteria in the organic nest material, although another possible explanation that merits further investigation could be an increase in permeability, leading to embryo dehydration.

Keywords Broad-snouted caiman · Egg pathologies · Egg mineralisation

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Introduction

Like their extant archosaurian counterparts, birds, all living crocodilians (cf. crocodylomorphs; alligators, crocodiles and gharials) normally lay regularly calcified eggs (Erben 1970; Jenkins 1975; Ferguson 1985; Webb and Smith 1987; Palmer and Guillette 1992; Thorbjarnarson 1996; Deeming and Unwin 2004; Campos et al. 2008). However, individuals living in captivity often produce eggs that are softer and less regularly calcified because they are fed a diet poor in calcium or because of the effects of pesticides and other local environmental factors (Erben 1970; Erben et al. 1979; Hirsch 2001; Poletta et al. 2009). Indeed, similarly formed poorly calcified eggs are common in domestic poultry

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(Vargas-Albarracin 2012) and pheasants (Sauveur and Reviers 1992) (e.g. Nathusius 1868; Asmundson 1931, 1933; Romanoff and Hutt 1945; Romanoff and Romanoff 1949; Sauveur and Reviers 1992), and defective eggs of abnormal thickness (double and multilayered shells) and with unusual ornamentation have been reported in turtles, birds, and even non-avian dinosaurs (Nathusius 1868; Romanoff and Romanoff 1949; Schmidt 1967; Erben 1970, 1972; Erben et al. 1979; Ewert et al. 1984; Hirsch 2001; Jackson and Varricchio 2003; Jackson et al. 2004; Fernández and Matheos 2011). Little, however, is known about the pathologies of extant crocodilian eggs (see comments in Erben 1972; Schlëich and Kastle 1988). Here, by restricting our study to a single living species for which we have collected comprehensive data, we ask: What are the characteristics of poorly mineralised *Caiman latirostris* eggs and what factors may have influenced their formation?

The eggshell microstructure of living crocodilians remains poorly described. Shell morphologies of *Alligator mississippiensis* (Ferguson 1982), *Crocodylus niloticus* (Zhao and Huang 1986) and *Alligator sinensis* (Grine and Kitching 1987) have been described, and Schlëich and Kastle (1988) briefly mentioned eggshells of *C. latirostris* in their atlas of scanning electron microscope (SEM) images. Weyenbergh (1876) discussed a record of an egg with an incomplete shell collected from an alligator nest, while more recently, Paz et al. (1995) described and compared eggshells from two Argentine caiman species (*C. latirostris* and *Caiman yacare*).

In this study, we discuss the broad-snouted Caiman (*C. latirostris*), a species found across western South America and presently considered a low conservation risk (Verdade et al. 2010; Larriera 2011; Larriera et al. 2008). Based on a comprehensive survey of nests and eggs of this crocodilian, we present, for the first time, a series of original observations of well-calcified and poorly calcified shell morphologies and document some causes of poorly calcified eggs and ornamented well-calcified eggs in the wild and captive individuals surveyed. The data we present are important for the design of future caiman egg monitoring strategies in the field and in captivity as we establish causes and patterns for shell abnormalities.

Materials and methods

We studied *C. latirostris* eggs collected between 2005 and 2011 by Proyecto Yacaré (Larriera 1990). Eggs were sampled from caiman nests in natural habitats and in the experimental breeding facility Estación Zoológica Experimental (EZE) in Santa Fe Province, Argentina. We removed eggshell samples from well-calcified (‘normal’) eggs at two distinct times: (1) during incubation and (2) after hatching. Shell samples were then compared with those taken from corresponding positions at the pole and equator of well-calcified *C. latirostris* eggs and noticeably less well-mineralised shells taken during the final period of incubation. Over the 6 years of this project, we surveyed more than 1,300 nests and tabulate basic data from 86 well-calcified (normal) eggs (13 different nests) alongside less well-mineralised (‘abnormal’) eggs from just eight nests (tabulated in Online Resource 1). Our definition of normal versus abnormal caiman eggs is based on these data (1,300 normal versus 13 abnormal nests observed over six field years); our data show that, overwhelmingly most often, *C. latirostris* eggs are well-calcified (tabulated in Online Resource 1) (Table 1).

We studied eggshell surface ornamentation and pores, shell structure exposed on the radial surface and mammillae on the inner surface from 7 to 15 times magnification using a binocular microscope (Nikon SMZ 645). Thirty-micrometre-thick eggshell thin sections were made from one poorly mineralised caiman egg and 18 well-calcified eggshells and imaged using a polarising light microscope (LabKlass model JPL-1350), with and without analyser, at four and ten times magnification. Additional well-mineralised and poorly mineralised caiman eggshells were imaged for this project using a scanning electron microscope with a field emission gun (SEM FEG Nano Nova 230).

Results

Normal well-calcified *C. latirostris* eggs are white, ornamented and oval in shape (Fig. 1a). They are slightly ellipsoid with a long axis that ranges from 5.95 to 7.60 cm in length (mean of our data, 6.3; N=34). The shorter axis of the caiman eggs in our sample ranges from 3.80 to 4.50 cm (mean, 4.09 cm; N=46) (Fig. 1a), and eggshell thickness (excluding ornamentation) varies from 0.36±0.07 mm (N=86) at the poles to 0.41±0.09 mm (N=86) at the equator. Ornamentation can increase these thicknesses 0.72±0.12 mm (N=86) and 0.73±0.10 mm (N=86), respectively (Fig. 1b).

Normal well-calcified *C. latirostris* eggs are opaque and have an ornamented external surface. This surface is covered with lacunae, tubercles and bridges built up from sharply separated and superimposed calcareous layers (Figs. 1b–d and 2b, c). Ornamentation is columnar in microstructure, giving the shell surface its characteristic ornamentation (Fig. 1). Columns are formed by concentric deposits of calcite that are expanded at different heights and connected to one another irregularly via expansions of other adjacent columns (Figs. 1b–d and 2b, c). These lacunae, the terminology of Schlëich and Kastle (1988), probably correspond with the areas of ‘crater resorption’, a term used by Mikhailov et al. (1996). Note that in their work,

Table 1 Data from all *C. latirostris* nest and egg data collected between 2005 and 2010. These data form the basis of our results and discussion (see text for details)

Year	Condition	Clutch size	Irregularly calcified eggs	Hatching success (%)	Nest no.
2005	Captivity	34	1	3 (8.8)	173
2007	Captivity	29	29	0 (0)	115
2008	Wild	34	1	2 (5.9)	127
2010	Captivity	47	47	4 (8.5)	47
2010	Captivity	13	13	5 (38.5)	51
2010	Captivity	36	36	0 (0)	61
2010	Wild	21	1	2 (9.5)	86[a]
2010	Captivity	47	47	0 (0)	10

[a]Eggs in this nest were partially shelled (see text for details)

however, Mikhailov et al. (1996) referred to the egg surfaces of crocodilians in general.

Based on our original observations, normal well-calcified *C. latirostris* eggs have an eggshell unit made up of a single ultrastructural zone (Figs. 1b and 2a, b) as well as a membrane that forms the inner part of the shell (Fig. 2d). This outer single calcareous layer has units formed from the irregular radial growth of tabular wedge-like crystals (Fig. 1b) with a basal plate group (rosette) (Fig. 3a); the organic core is absent (Figs. 1b and 2b, d). Pore channels are also present on the external surface of normal well-calcified caiman shell and are points for resorption. These channels are most evident when shells are viewed with a binocular microscope under transmitted light (Figs. 1b–d and 2b) and are most numerous at the equatorial regions of the eggs. The shell membrane, in contrast, is composed of two different parts; the outer layer is made up of randomly oriented fibres (Figs. 2d and 4b), while its innermost surface has a thin, amorphous and non-fibrous, surface-limiting layer (Fig. 2d). Figure 3 shows the inner surface of the calcareous layer with a rosette evident (Fig. 3a), the rest of the flexible layer detached, and detail of the fibrous layer of the membrane (Fig. 3b). Over the course of our 5-year sample, well-calcified *C. latirostris* eggs had a mean hatching success of 75 % (Table 1).

We observed two types of abnormality in our sample of well-calcified *C. latirostris* eggs: calcite grain aggregates and concentric wrinkles occurring around one egg pole

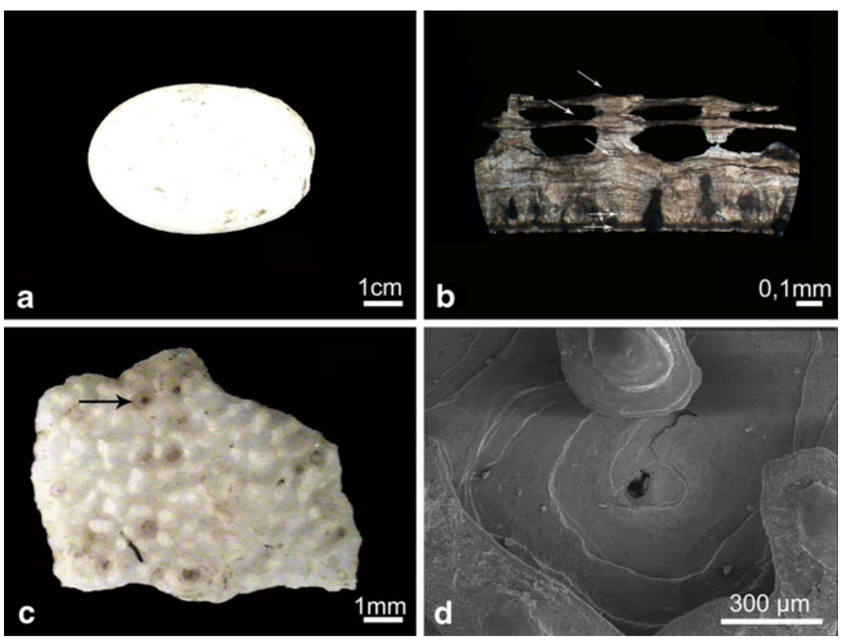


Fig. 1 Normal eggs and eggshell of *C. latirostris*. **a** Well-calcified oval white egg. **b** Thin section image of eggshell; in this image, the *upper arrow* indicates the outer portion of the eggshell and the ornamentation consisting of shaped 'towers', while the *middle arrow* indicates connections between towers. The *third arrow* shows growth lines of calcite deposition that form the shell units. The *fourth arrow* points to a wedge-like subunit, while the *lowest arrow* shows the base of the unit that consists of a rosette with no evidence of an organic core. **c** Outer surface of well-calcified caiman eggshell viewed with a light microscope; *black arrow* indicates craters with pores and towers of ornamentation. **d** Surface view under SEM to show lacunae in detail with characteristic rings of degradation and a pore

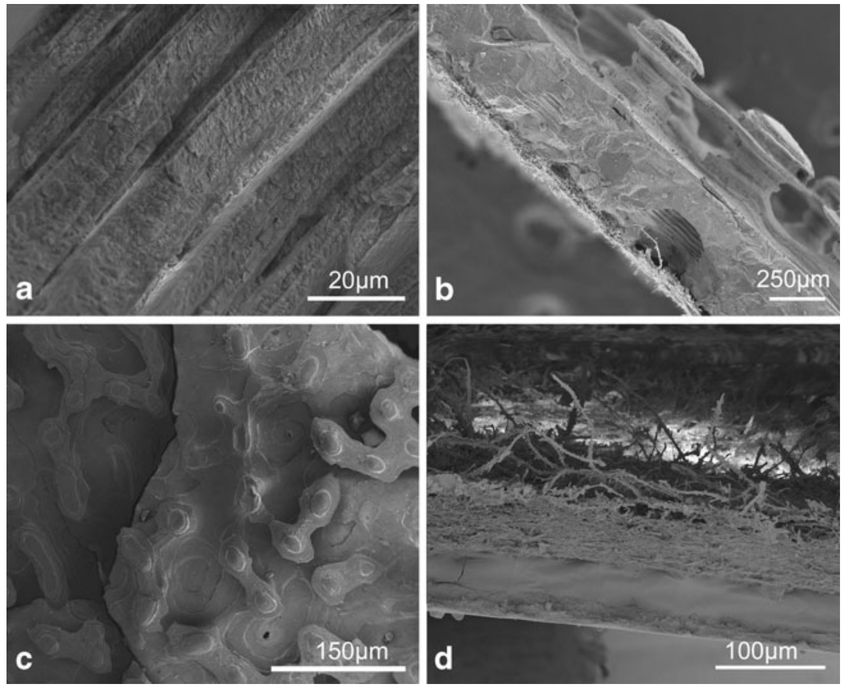


Fig. 2 SEM images of well-calcified *C. latirostris* eggshell. **a** Radial view showing the superimposed tabular crystals that form the single ultrastructural zone of the eggshell. **b** Radial view of eggshell showing adjacent units that form a continuous wall interrupted only by pore channels and towers that form external ornamentation. **c** External surface showing the towers and lacunae that make up the ornamentation of the eggshell. **d** Innermost membrane formed by two layers. Of these, the upper layer is composed of randomly oriented fibers and has an innermost surface with a thin amorphous, non-fibrous layer

(Fig. 4). SEM imaging shows that the calcite that makes up these grains and wrinkles is not tightly interlocked but irregularly deposited (Fig. 4c, d). A small number of our wild collected caiman clutches contained eggs with poorly calcified shells (Table 1). However, it is difficult to determine the exact proportion of these abnormal eggs because excess calcification (abnormal ornamentation) is often lost during incubation (in both natural and artificial situations). Indeed, the abnormal ornamentations we report are attached to the outside of shells and are very fragile, so minimal contact with the nest material is sufficient to knock them off the surface during incubation. Thus, in any given nest, only a few eggs may retain these structures. In general, well-calcified eggs—even if formed abnormally—have a good chance of hatching success because excess calcification structures are often lost during incubation.

The thickness of the poorly mineralised *C. latirostris* eggs in our sample varies between 0.35 and 0.40 mm, with a pore density of $\leq$0.03 pores/mm^2. Radial thin sections and observations under binocular microscope show that these poorly mineralised eggshells lack surface ornamentation and that there are clear gaps within the shell structure (Fig. 5b, c). Columnar crystals that normally form beneath the ornamentation are entirely absent in these eggs (Fig. 5a, b), and eggshells contain gaps that weaken the shell and interrupt the normally harder and continuous wall (Fig. 5c). Poorly mineralised *C. latirostris* eggs are very fragile. We recorded nests containing recently laid eggs with membranes already detached and most shells broken (Fig. 5a). No embryos survived in these extreme cases, while nests with a reduced number of thin eggshells merely had a lower hatching success rate (typically 9 % or less) (Table 1).

Discussion

The limited number of previous studies to consider the formation of abnormalities in crocodilian eggs have

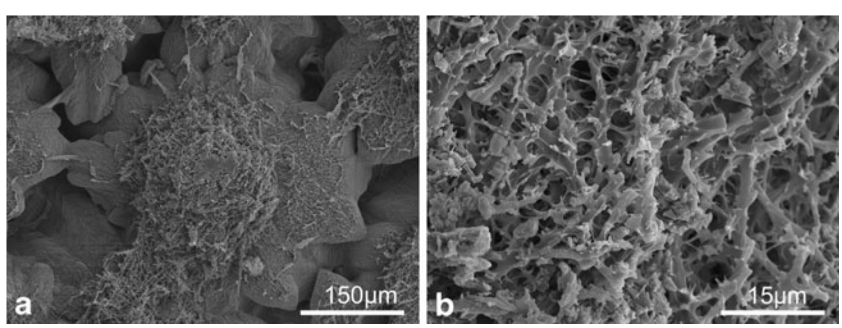


Fig. 3 SEM images to show details of the structure of well-calcified *C. latirostris* eggshell. **a** Basal plate group with a rosette centrally. **b** Inner view of the units and details of protein fibers that are attached to the base of the eggshell unit. Note that these fibers have a roughly reticulate pattern

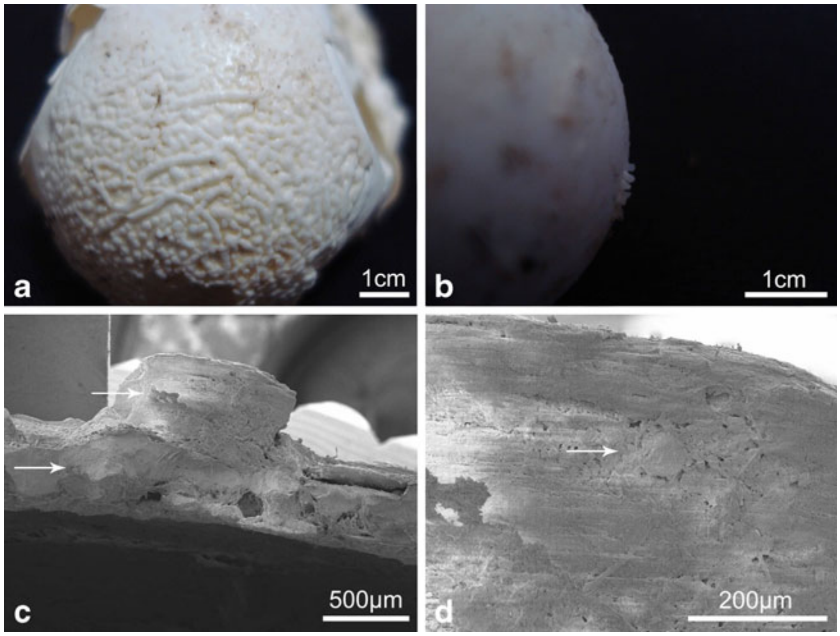
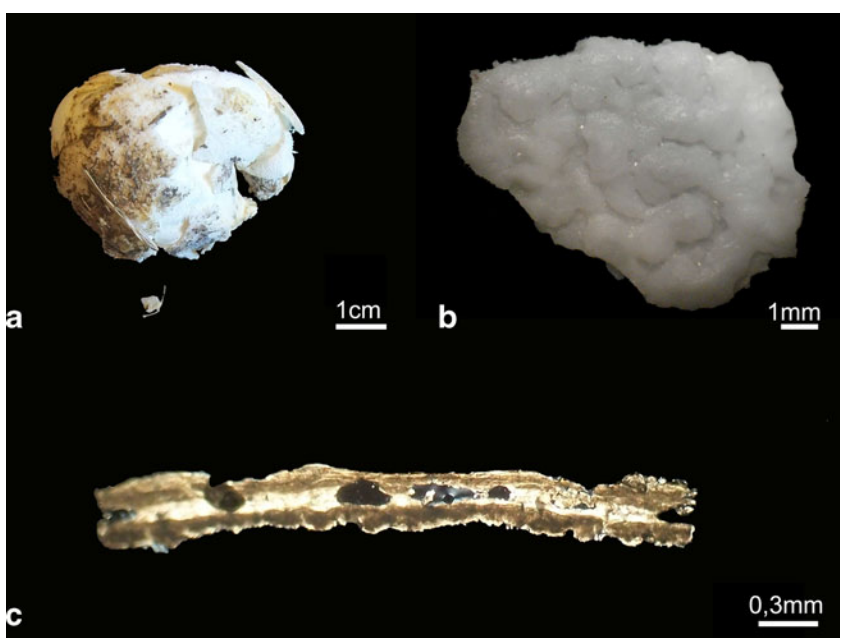


Fig. 4 Well-calcified *C. latirostris* eggs with abnormal calcification patterns. **a** Calcitic grains deposited at the pole of the egg. **b** Grains located at the pole of the egg. Note that the surface of this egg has already lost most grains leaving a smooth surface. **c** Egg with abnormal ornamentation in the forms of wrinkles (seen in radial view under SEM). The *upper arrow* denotes the massive structure of the egg pole, while the *lower arrow* indicates the continuous wall of the eggshell. **d** Zone shown in **a** under higher magnification shows loosely packed calcite crystals, porous areas and laminations that differ from normal ornamentation. The *arrow* shows another loosely packed zone on the boundary between shell units

attributed low hatching success and embryonic death by suffocation in *A. mississippiensis* and *A. sinensis* either to inadequate numbers of eggshell pores or to the presence of calcium deposits blocking pore entrances (Wink et al. 1990; Wink and Elsey 1994). Our observations do not confirm these observations in the specific case of *C. latirostris*: well-mineralised eggs in this species have good survival rates, even if abnormally formed. Most of the time, in *C. latirostris*, our observations indicate that increases in hardness result from over-calcification of the shell.

Kellogg et al. (1981) noted that the eggshells of alligators 'show progressive crystal dissolution, with the production of

Fig. 5 Poorly calcified *C. latirostris* eggs. **a** Complete egg with poorly calcified eggshell and fractures on the shell surface. **b** External surface of eggshell without typical ornamentation and without well-calcified normally continuous outer layer. **c** Thin section image through soft caiman eggshell showing poorly compacted units with gaps. As can be seen from these images, this shell has a poorly calcified structure and is highly fragile



concentrically stepped erosion craters with characteristic rings, as incubation progresses'. Our study confirms that this is also the case in *C. latirostris*; in this species, each ring is one crystal high (Fig. 1d). Numerous microorganisms can produce acidic metabolites as fermentation products in decaying nest vegetation; these acids, in combination with carbonic acid (generated by the hydration of carbon dioxide), will act dissolve calcite crystals in the outer densely shell layers (Ferguson 1981). This effect could enlarge surface defects in the erosion craters observed (Fig. 1c, d). Indeed, microbial degradation of nesting materials may explain the loss of hard calcium ornamentation in some eggs, and in our observation, it can completely disappear by the end of incubation.

Although shell over-calcification in *C. latirostris* may not compromise the survival of any given embryo (see below), it does represent an additional energetic cost for the female. In the less well-mineralised *C. latirostris* eggs observed, a much lower mean hatching success (ca. 9 %) is most likely due to microbial infections from organic nest materials Kellogg et al. (1981). Inability to limit water uptake because of increased shell permeability has also been reported to lead to the death of crocodilian embryos (Ferguson 1985; Packard 1991).

Reasons for abnormal eggshell development in reptiles remain debated. Hirsch (2001) suggested that 'pathological' eggshells are an indication of abnormal biological or environmental conditions. In other words, if a shell is thinner or thicker than normal for a given species, it disturbs the delicate equilibrium for the successful development of the embryo (Ar et al. 1979). Perhaps more pertinent egg and eggshell abnormalities in birds have been linked to agricultural pollutants (such as cadmium or DDT) which are known to produce hormonal changes that interfere with calcium metabolism (Erben 1972). These effects on crocodilian eggs are much less well understood, but we can distinguish them in our sample because captive caiman at EZE were fed once a week with a specially designed food that is free of chemical residues, while those living in natural habitats would have been affected by any herbicides in agricultural runoff.

One systemic herbicide in widespread use within the habitat of *C. latirostris* is glyphosate (*N*-phosphonomethyl glycine), used for non-selective weed control in agriculture and non-agricultural activities. Poletta et al. (2009) reviewed the environmental risk associated with glyphosate contamination within the habitat of *C. latirostris*. During the 1990s, there was a significant agriculture transformation in developing countries such as Argentina, driven by the adoption of transgenic crops (soy, corn) and 'no-tillage' systems. Nowadays, this agricultural change is still affecting the natural environments inhabited by alligators and caiman (Poletta et al. 2009).

It thus seems very possible that herbicides used in agriculture, which are known to cause genetic damage (Poletta et al. 2009), may be one key factor affecting the structure of caiman eggshells. Another studies on captive birds have inferred that egg decalcification (poorly calcified) could also be a consequence of poor diet, caused by a lack of calcium in the female, or other abnormalities in the female reproductive system (Hirsch 2001; Ferguson 1985, p. 359). Environmental factors inducing stress may cause birds to retain eggs in their oviducts (Romanoff and Romanoff 1949; Erben 1970; Erben et al. 1979), and weak calcification may also be due to a shortage of calcium in the diet (Ferguson 1985, p. 359) of the mother brought on by the stress of captivity. It is thus very important when designing monitoring strategies for crocodilian eggs—both in the field and in captivity—to establish both patterns of abnormalities (as we have done for *C. latirostris*) and possible causes (we have presented some suggestions in this paper for *C. latirostris*). The baseline information we present here is the first of its kind for *C. latirostris* and will provide a basis for a better understanding of the anomalies observed in fossil crocodilian and possibly other reptilian eggs.

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