



FIRST RECORD OF AN AMNIOTIC EGG FROM THE ROMUALDO FORMATION (LOWER CRETACEOUS, ARARIPE BASIN, BRAZIL)

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ABSTRACT – Fossil amniotic eggs have great informative potential, especially regarding reproductive and evolutionary aspects of vertebrates. However, there are only few intact specimens or with fossilized embryos within, and the rare reported cases are mostly related to dinosaurs. In Brazil, the records of these ichnofossils are practically restricted to the Bauru Basin. This research aims to describe the first amniotic egg found in carbonate concretions in the Romualdo Formation, adding information to the study of these fossils and to the paleontological context of the basin. The specimen was collected at the Sítio Pé da Serra do Félix, in the Municipality of Simões, Piauí State, Brazil. The methodology employed was based on Scanning Electron Microscopy, Energy Dispersive Spectroscopy and Optical Microscopy techniques, as well as computed tomography analyses. The morphological and microstructural characteristics of the shell suggests the identification of the fossil as a crocodylomorph egg. This specimen differs from other fossil eggs assigned to the aforementioned group by its small size and considerably thick shell. The tomographic sections revealed possible basic structures of an embryo inside the egg, suggesting that this is the first fossilized egg with a crocodylomorph embryonic trace found in the world.

Keywords: ichnofossil, amniotic egg, embryo, Crocodylomorpha, Aptian, Romualdo Formation.

RESUMO – Ovos amnióticos fósseis possuem um grande potencial informativo, especialmente em relação aos aspectos reprodutivos e evolutivos dos vertebrados. Contudo, existem poucos exemplares intactos ou dotados de vestígios embrionários em seu interior e os raros casos registrados são em sua maioria relacionados a dinossauros. No Brasil, os registros desses icnofósseis estão praticamente restritos a Bacia Bauru. O presente trabalho objetiva descrever a primeira ocorrência de um ovo amniótico encontrado em concreções carbonáticas da Formação Romualdo, acrescentando informações ao estudo desses fósseis e ao contexto paleontológico da bacia. O espécime foi coletado no Sítio Pé da Serra do Félix, no Município de Simões, Piauí, Brasil. A metodologia empregada baseou-se nas técnicas de Microscopia Eletrônica de Varredura, Espectroscopia por Energia Dispersiva e Microscopia Óptica, bem como análises de tomografia computadorizada. As características morfológicas e microestruturais da casca sugerem que o fóssil se trata de um ovo de crocodilomorfa. O espécime analisado difere de outros ovos fósseis atribuídos ao referido grupo taxonômico pelo tamanho reduzido e casca consideravelmente espessa. As seções tomográficas revelaram possíveis estruturas básicas de um embrião no interior do material, sugerindo que se trata do primeiro ovo fossilizado de crocodilomorfa com restos embrionários do mundo.

Palavras-chave: icnofóssil, ovo amniótico, embrião, Crocodylomorpha, Aptiano, Formação Romualdo.

INTRODUCTION

The formation of an ichnofossil involves three main factors: the producer, the preserved behavior pattern, and the substrate and sediment types that allowed its preservation. The relevance of the study of an ichnofossil is the aid in paleoenvironmental and paleoecological interpretations of organic activities in a given geological time, as well as highlighting the behavior of different organisms and the sedimentation conditions in the past (Souto, 2017). As such, fossilized amniotic eggs are very important as they contribute to elucidate the reproductive and evolutionary aspects of vertebrates, especially if they harbor embryos. However, these fossils are very rare due to their fragility, and most of the record is of dinosaurs, with reports of nests, complete or fragmented eggs, and also the occurrence of embryonic integument (Mikhailov, 1997; Chiappe *et al.*, 1998; Chiappe *et al.*, 2001; Grellet-Tinner *et al.*, 2004, 2012; Srivastava *et al.*, 2015).

In Brazil, fragmented eggshells and complete eggs of dinosaurs, crocodylomorphs, chelonians and birds documented from Cretaceous rocks occur mainly in the south-east region, in the sediments of the Upper Cretaceous Bauru Basin (Marsola, 2013; Marsola *et al.*, 2014a,b; Magalhães-Ribeiro, 2017). In the northeast, there are only occurrences of putative eggshells of uncertain taxonomic affinities from the Aliança Formation (Upper Jurassic, Jatobá Basin) in the Pernambuco State (Silva *et al.*, 2012), of eggshells ascribed to dinosaurs from the Itapecuru Formation (Lower Cretaceous, Parnaíba Basin) in the Maranhão State (Vicalvi *et al.*, 1993), and of a complete crocodylomorph egg from the Crato Formation (Lower Cretaceous, Araripe Basin) in the Ceará State (Magalhães-Ribeiro *et al.*, 2011).

The Crato and Romualdo formations, lithostratigraphic units that compose the structural framework of the Araripe Basin, are two of the most important paleontological sites in Brazil (Viana & Neumann, 2002). However, despite the diverse paleobiota already documented for the Romualdo Formation, especially of vertebrates (Maisey, 1991), there was still no record of amniotic eggs for this deposit. Thus, this paper aims to analyze the first record of a fossil egg, possibly from Crocodylomorpha, collected in the sedimentary layers of the Romualdo Formation.

GEOLOGICAL SETTING

Located in north-eastern Brazil, the Araripe Basin originated from processes associated with the rifting of the supercontinent Gondwana, which led to the opening of the South Atlantic Ocean, consequently forming the South American and African continents (Matos, 1992; Assine, 2007). Between the lithostratigraphic units that constitute this basin, the Santana Group is the most important from a paleontological point of view as it has two Lagerstätte deposits: the Crato and Romualdo formations (Kellner, 2002; Viana & Neumann, 2002; Carvalho & Santos, 2005).

The Romualdo Formation, composed by basal sandstones overlapped by greenish marls and fossil-rich calcareous concretions, marks an important marine ingressão in the northeast of Brazil during the late Aptian (Custódio *et al.*, 2017; Teixeira *et al.*, 2017). Its fossiliferous association includes plants, invertebrates, and mainly vertebrates, with a predominance of the Osteichthyes and Pterosauria groups, but including Testudines, Crocodylomorpha and Dinosauria as well (Santos & Valença, 1968; Kellner, 1987; Maisey, 1991; Viana & Cavalcanti, 1991; Martill, 1993; Kellner, 1998, 2002; Kellner & Tomida, 2000; Oliveira & Kellner, 2007; Oliveira & Romano, 2007; Lima *et al.*, 2012; Polck *et al.*, 2015; Custódio *et al.*, 2017). From a mass mortality event (Martill, 1988; Maisey, 1991; Viana & Sial, 1999), the eodiagenesis of concretions allowed the extraordinary preservation of these fossils, including fragile materials (Kellner, 2002).

The studied material was collected on the western border of the Araripe Basin, in the state of Piauí, in the upper part of the Romualdo Formation. This stratigraphical level is recognized in many localities in the Araripe Basin and is marked by the occurrence of coquinas, indicating the top of the Aptian in the proximal marine environment (Viana & Long, 1997; Assine *et al.*, 2014; Custódio *et al.*, 2017). The stratigraphic section of the local outcrop shows a sequence of layers, approximately six meters thick of the total exhibition, consisting of greenish to brownish shales, with concretions and presenting lenses (upper meter), both carbonated, placed discordantly on the crystalline basement. At the site, several concretions containing coprolites and fish remains were also collected. In addition, the upper levels presented bioturbations and other fossils that are still being studied by algal and marine

invertebrate specialists. The fossil here studied was collected 2.6 m from the top of the Romualdo Formation (Figure 1).

MATERIAL AND METHODS

The studied material consists of one isolated egg preserved in a calcareous concretion. The specimen was collected in 2015 at the locality Sítio Pé da Serra do Félix (7°35'33.0"S/40°44'36.1"W, datum SIRGAS 2000), in the Municipality of Simões, southeast of the Piauí State. The project authorization number is COPAL/ANM 000.822/2015. The fossil material is deposited in the fossil collection of the Museu Dom José (MDJ), located in the Municipality of Sobral, in the State of Ceará, with the designations of MDJ Ic-069a and MDJ Ic-069b (Figure 2), corresponding to the part and counterpart of the same concretion.

The fossil egg was enveloped by a calcareous concretion. For this reason, only small fragments were removed from its shell and subjected to the Scanning Electron Microscopy (SEM), Dispersive Energy Spectroscopy (EDS) and Optical Microscopy (OM) techniques, for visualization and analysis of morphostructural characteristics, thus maintaining the integrity of the specimen.

Three fragments, in different histological sections (external, internal, and radial surface), were observed using SEM InspectS50-FEI and with EDS at the Analytical Center of the Universidade Federal do Ceará (UFC). Only one slide with the shell in a radial section was prepared in the Laboratório de Laminação (LAMIN). Afterwards, the slide was analyzed using high resolution petrographic microscopy (Nikon 3 Di, 100x visual acuity) with a connected camera in the Laboratório de Microscopia Eletrônica (LME). Both laboratories belong to the UFC Geology Department.

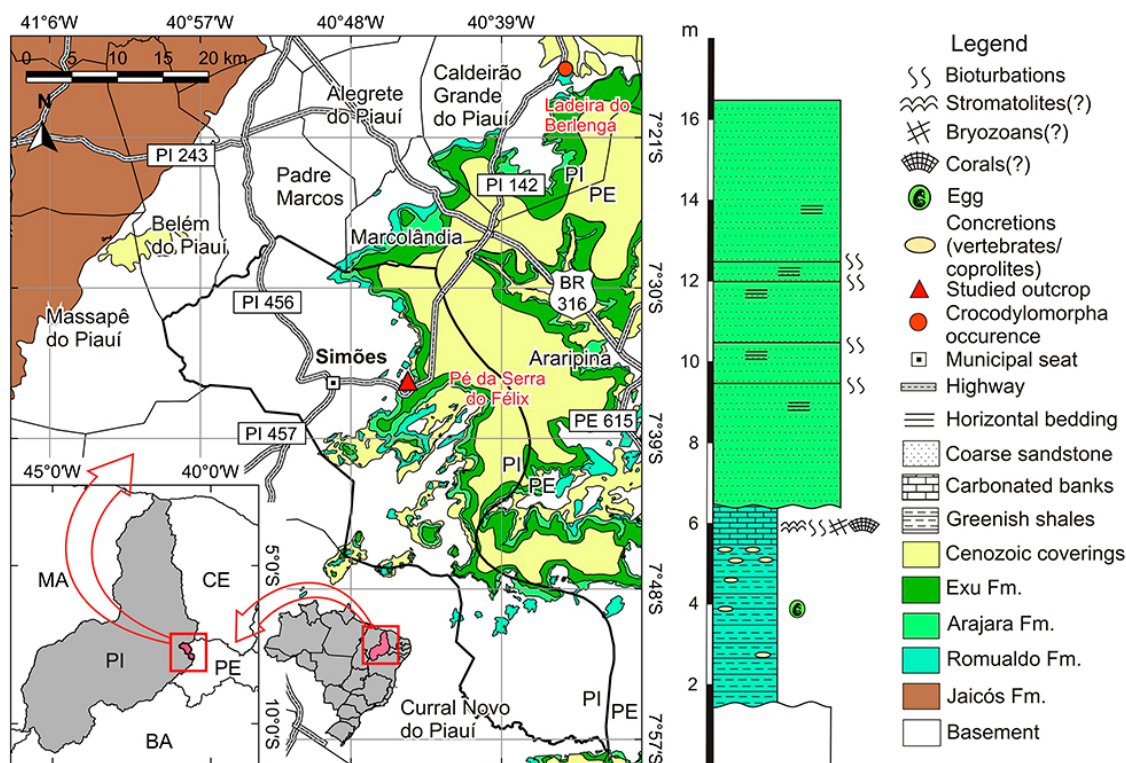


Figure 1. Location of the studied area and a local stratigraphic section with the egg occurrence horizon. Araripe Basin limits based on data provided by the Brazilian Geological Survey (CPRM Companhia de Pesquisa de Recursos Minerais: geosgb.cprm.gov.br). Processed with the QGIS software by J.V.P. Moreira in 2018.

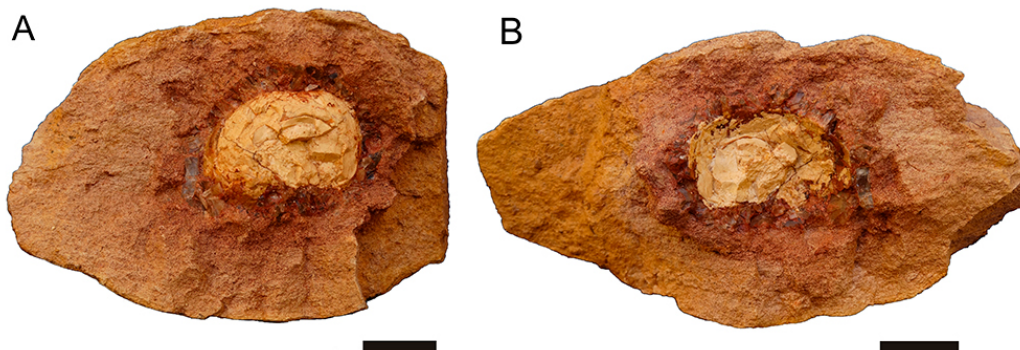


Figure 2. Fossil egg described in this study. A, MDJ Ic-069a; B, MDJ Ic-069b. Scale bars = 20 mm.

In addition, the specimen underwent a computed tomography in a private human radiological clinic. The use of this technique allows inferences about the interior morphology of the egg (Magalhães-Ribeiro, 2007). The examination was performed on a four-channel CT (Toshiba, model Alexion, Tokyo, Japan). The parameters used in this examination were: 120 kV and 200 mA, 2 mm cross sections, and bone and soft tissues filters, whose images were reconstructed in dorsal and sagittal plane. In order to increase the contrast differentiation of the images obtained by tomography, these were stored as Digital Imaging and Communications in Medicine (DICOM) format and then transferred to the computer program of a 1.5 Tesla magnetic resonance imaging device (MRI–Siemens, Magneto Essenza, Berlin, Germany). Thus, the images were analyzed in different filters provided by the program with the objective of choosing the filter that best characterized the internal structures of the fossilized egg. Taken from a specific program for the analysis of radiological examinations (ClearCanvas Workstation – version 2.0 SP1; Toronto, Canada), the images processed by the aforementioned magnetic resonance computer program were evaluated in transverse, dorsal and sagittal planes, in addition to variations of brightness, contrasts and densities based on the Hounsfield Unit.

For a better identification of the entire tomographic anatomy of the specimen, a tomographic examination was also performed on an egg of the extant species *Caiman latirostris* (CHUFC C048, registered in the UFC Herpetology Collection), which was in its final stage of embryonic development. These images were evaluated in a soft tissue filter in sagittal plane. The analyses were approved by the Ethic Committee on Animal Use of the Universidade Federal do Ceará (CEUA-UFC), CEUA protocol number 9806051018. After the computed tomography scan, the egg was dissected and the embryo (*C. latirostris*) was removed from the egg. The sagittal section was performed on the midline, corresponding to the tomographic image of the fossil specimen.

RESULTS AND DISCUSSION

Description and morphological comparison

MDJ Ic-069 presents a slightly elliptical shape, measuring 26.02 mm and 18.15 mm in its major and minor axes, respectively. The dimensions of MDJ Ic-069 are small compared to other crocodylomorph eggs from the Brazilian Cretaceous, such as a specimen from the Crato Formation (Magalhães-Ribeiro *et al.*, 2011), or those described by Oliveira *et al.* (2011) and Marsola *et al.* (2016) from the Adamantina Formation, in the Bauru Basin. On the other hand, its size resembles eggs from the Araçatuba Formation in the Bauru Basin ascribed to *Mariliasuchus amarali* (Magalhães-Ribeiro *et al.*, 2006), as well as from eggs reported by Novas *et al.* (2009) from the Bolivian Cretaceous (Table 1).

The eggshell thickness is about 0.45 mm, which is quite significant when compared to the egg of the Crato Formation (Magalhães-Ribeiro *et al.*, 2011). It is more similar to the shell thickness in eggs of the living species *Paleosuchus*

Table 1. Comparative dimensions and shell thickness of some crocodylomorph eggs.

Lithostratigraphic units	Egg dimension (major axis X minor, in mm)	Shell thickness (mm)
Crato Formation (Brazil)	43 x 29	0.1
Adamantina Formation (Brazil)	72.6 x 42	0.13–0.15
Araçatuba Formation (Brazil)	65 x 36	0.15–0.25
	35 x 45	0.24–0.36
	30 x 50	
Lameta Formation (India)	68 x 44	0.43–0.47
Cajones Formation (Bolivia)	30 x 16	0.2
Romualdo Formation (Brazil)	26.02 x 18.15	0.45

palpebrosus, whose average thickness is 0.41 mm (Marzola *et al.*, 2015) and the specimens described by Srivastava *et al.* (2015) for the Indian Cretaceous, which have average thickness between 0.43–0.47 mm. However, a shell fracture was observed through the tomography, in the left lower portion of the egg. Thus, due to this fracture and the potential loss of fluid from within the egg, it is difficult to ascertain whether the dimensions of the specimen have changed or not. In addition, this fracture may have caused changes in eggshell thickness.

The spectroscopic analysis in the eggshell basic units revealed the presence of calcium carbonate (CaCO_3), which is the predominant matter of the mineralized eggshell (Kohring, 1995) and, in some points, of calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$), as well as the presence of Barium (Ba) and Sulphur (S) (Figure 3). The latter two elements may be related to decomposition processes in the marine environment (Dehairs *et al.*, 2000; Reitz *et al.*, 2004).

The eggshell outer surface is smooth, presenting low undulations, with pore openings randomly distributed. Such structures may be interconnected, forming larger openings, similar to the material described by Moreno-Azanza *et al.* (2015) (Figures 4A–B). These pore openings were also observed on the inner surface, although it is not possible to measure the quantitative data of these structures due to the restricted analyzed material (only one sample), as previously mentioned. In the radial section of the shell, the basic units individually exhibit the typical crocodylomorph pattern, consisting of polycrystalline units of calcite in the form of compact wedges, with small interstices in its bulbous base (Figures 4C–D) (*e.g.* Mikhailov, 1997; Oliveira *et al.*, 2011; Marsola, 2013; Moreno-Azanza *et al.*, 2015; Russo *et al.*, 2017). Since the crystals are not visualized through the entire radial section, a certain limitation was verified when observing these structures. In addition, the calcite layers or tabular microcrystallisations that have developed along the basic units are poorly highlighted. The configuration of the basic unit of the shell is much more evident using optical microscopy, which reaffirms the aforementioned descriptions (Figures 4E–F). However, with only one single sample to analyze, it was impossible to determine more details. The tabular

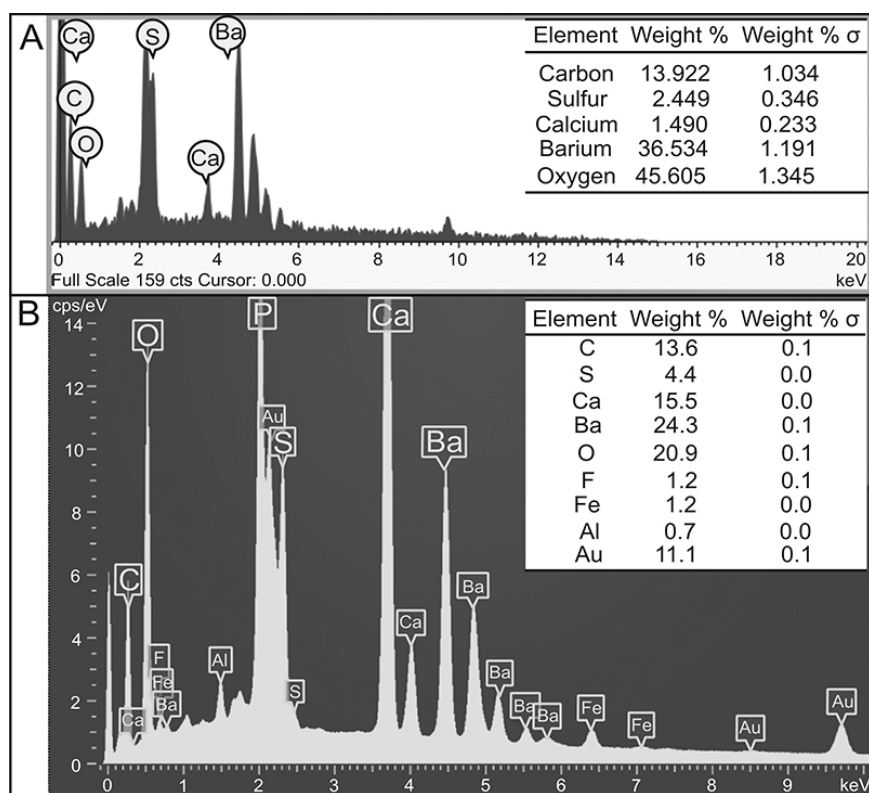


Figure 3. Spectrum of the elements identified in the eggshell. **A**, calcium carbonate; **B**, calcium phosphate.

microcrystallization is present in the crocodile eggshell units (Erben, 1970; Ferguson, 1982; Mikhailov, 1997) and is used as a diagnostic feature for the identification of crocodylomorph fossil eggs.

It is worth noting that the analysis of microstructural characters related to the crystalline structure of the egg shell, composed of the basic units of the shell and the pore system, is essential to associate the fossil egg to the producing animal group, since each amniote vertebrate taxon exhibits a characteristic pattern of this structure (Magalhães-Ribeiro, 2017). Thus, it is possible to make taxonomic assignments of these ichnofossils based on the microstructure of the eggshell (Mikhailov *et al.*, 1996; Magalhães-Ribeiro, 2007). In the fossilized or recent eggs of crocodylomorph, the characteristics observed in the shell microstructure are consistently different from those of other amniotes, thus allowing a conclusive taxonomic attribution (Russo *et al.*, 2017).

In the case of MDJ Ic-069, the limitation of samples analyzed in both SEM and optical microscopy hindered a more accurate diagnosis. Thus, the studied specimen is tentatively associated with crocodylomorph, based mainly on the configuration of the basic shell units, in addition to the other morphological characteristics presented.

Tomographic data

In the tomographic images of MDJ Ic-069, an embryo-like internal structure could be detected. The study of these findings is usually based on the observation of anatomical patterns in living taxa of the same group, which aim to explain and correlate the existent structures in the fossil specimen and

the degree or type of its development (Azevedo *et al.*, 2000; Andrade, 2005; Balanoff *et al.*, 2008). Thus, the tomographic image of both the fossilized specimen and the egg of the extant species, as well as the anatomical section of the respective embryo (*Caiman latirostris*), were anatomically correlated. This comparison allowed us to suggest an embryo-shaped image in the sagittal plane, as well as specific structures such as: eggshell, brain cavity, vertebral column and abdominal region (Figure 5).

The densities of the main regions present in the fossilized egg were quantified according to the Hounsfield Unit (Cogbill & Ziegelbein). This allows for the indication of more specific regions of the supposed embryo, according to density proportions corresponding to embryonic structures such as: skull, vertebrae and abdominal region. However, other regions of the fossilized embryo body and some anatomical details could not be identified due to the quality of the tomographic images, the initial state of ossification, or the small size or wear of anatomical structures (Schweitzer *et al.*, 2002; Balanoff *et al.*, 2008).

In the case of MDJ Ic-069, the taphonomic processes probably caused a change in the conservational state of the egg. Together with the substitution and mineralization of the soft tissues, this may have contributed to the lack of definition and to the increase of density of the caudal portion of the embryo, as well as to the loss of anatomical details such as the temporal cavities identified in crocodylians. Thus, it was not possible to specify the tomographic anatomy and to correlate it with the macroscopic anatomy of the crocodylomorph species of the Romualdo Formation – *Araripesuchus gomesii*

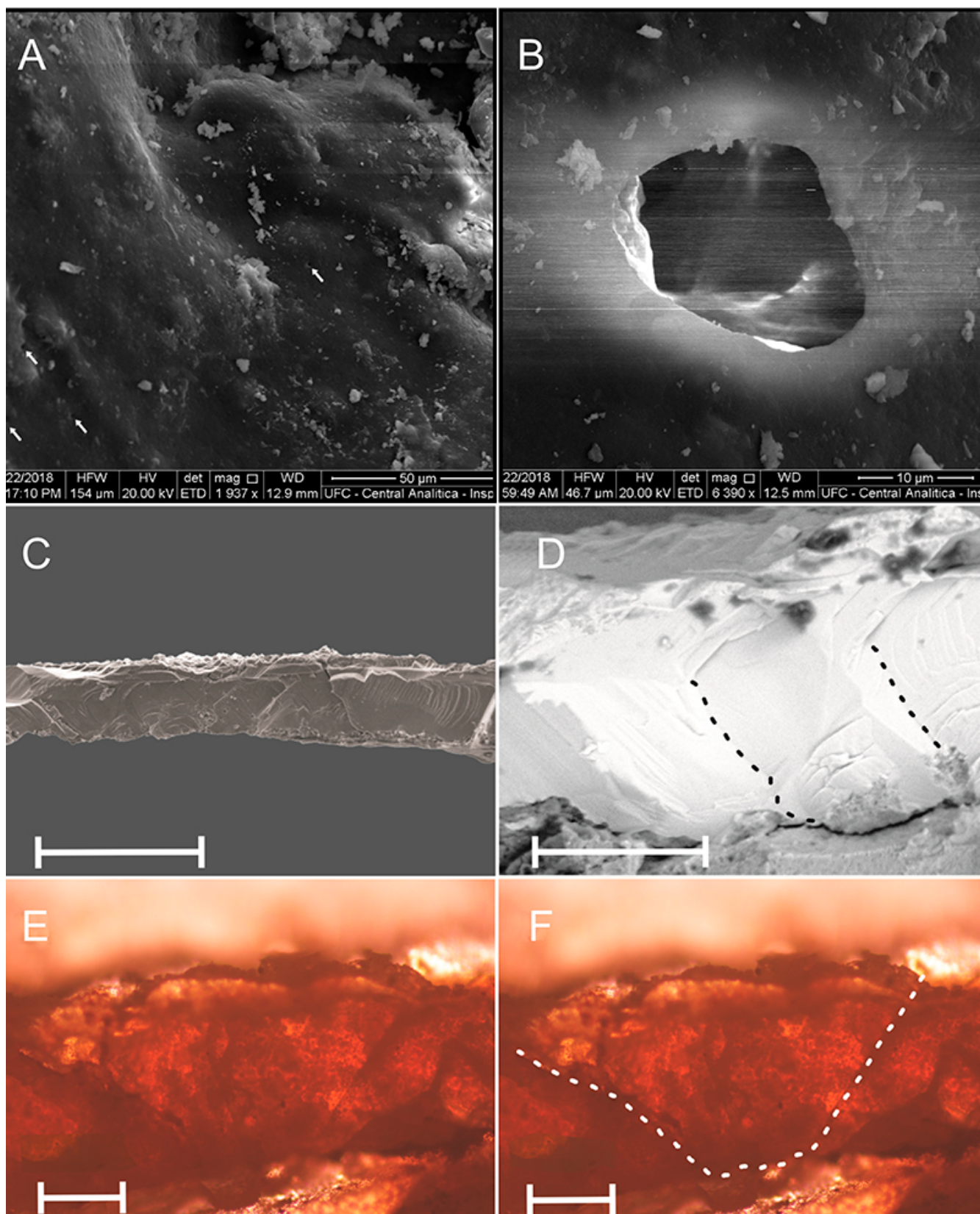


Figure 4. Microscopy analysis in MDJ Ic-069. Scanning Electron Microscope. **A**, outer surface of the shell exhibiting undulations, with pore openings randomly distributed (arrows); **B**, openings caused by interconnected pores; **C–D**, radial section of the shell; the lines indicate the boundaries between each unit of the shell. Optical microscope; **E–F**, configuration of the basic shell unit and its outline. Scale bars: C, E–F = 200 µm; D = 50 µm.

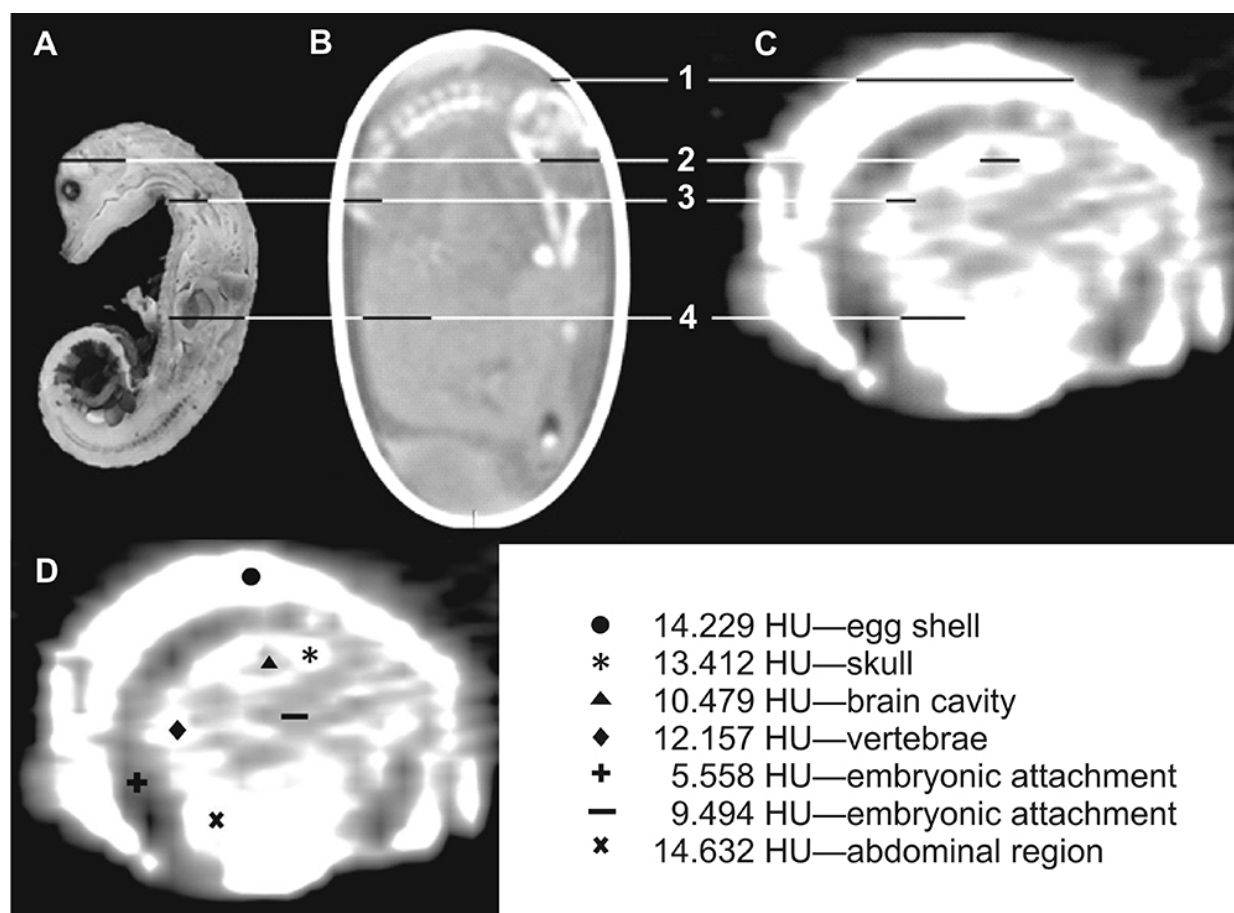


Figure 5. Correlation of the main anatomic characteristics of a present crocodylian embryo (*Caiman latirostris*) and MDJ Ic-069. **A**, sagittal section of *C. latirostris*; **B**, tomographic image of *C. latirostris* still inside the egg; **C**, tomographic fossil image (1, eggshell; 2, skull; 3, vertebral column; 4, abdominal region); **D**, approximate values of the densities in Hounsfield Unit (HU) and suggestions of the involved structures of the specimen.

or *Itasuchus camposi* (Price, 1959; Kellner, 1987; Riff *et al.*, 2012).

Even though it is not possible to identify the new discovery taxonomically, it is worth noting the presence of *Araripesuchus gomesii* in the same strata, in a locality about 38.5 km away from where the egg was found (Figure 1). Although suggestive, it is premature to assign this species as a possible producer due to the absence of osseous elements in association with the fossil egg, as established by the taxonomic confidence levels proposed by Grellet-Tinner (2005).

CONCLUSION

The analysis of the morphostructural characteristics observed in the shell of MDJ Ic-069 allowed us to present the first possible record of crocodylomorph egg from the Romualdo Formation in the Araripe Basin. This specimen differs from other fossil eggs attributed to the Crocodylomorpha by its small size and the considerably thick shell. Possible basic anatomical structures of a body embryo were recognized. Thus, MDJ Ic-069 could be the first fossil egg in the world with embryonic remains attributed to crocodylomorphs.

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