The eggshell features and clutch viability of the broad-snouted caiman (*Caiman latirostris*) are associated with the egg burden of organochlorine compounds

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ABSTRACT

Organochlorine compounds (OCCs) are toxic and have been identified as endocrine-disrupting chemicals (EDCs). The broad-snouted *Caiman* (*Caiman latirostris*) is an oviparous species widely distributed in South America with potential to accumulate OCCs. The eggshell is formed during passage of the eggs through the oviduct. Since the oviduct is a target of hormone actions, exposure to OCCs could modify eggshell quality, thus affecting clutch viability. Eight clutches were collected from wetlands of Parana River tributaries, in north-eastern Argentina. Two to four eggs per clutch were used to establish the burden of OCCs, eggshell thickness and eggshell porosity. The remaining eggs were incubated in controlled conditions. Ten days after hatching, hatchling survival was assessed. Organochlorine pesticide residues (OCPs) were found in all clutches, while polychlorinated biphenyls (PCBs) were present in all but one clutch. The principal contributors to the OCP burden were members of the DDT family and oxychlordane. Eggshell thickness was $400.9 \pm 6.0 \, \mu m$ and, unexpectedly, no association between eggshell thickness and the OCC burden was found. The number of pores in the outer surface was 25.3 ± 4.3 pores/cm^2. A significant inverse correlation between porosity and OCC burden was found (Pearson $r = -0.81$, $p = 0.01$). Furthermore, a decrease in caiman survival with decreased pore density was observed (Pearson $r = 0.73$, $p = 0.04$). Our findings highlight another potential negative impact of current and past use of OCCs on wildlife species.

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1. Introduction

Organochlorine compounds (OCCs), such as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), are persistent lipophilic chemicals identified as endocrine-disrupting chemicals (EDCs) (Colborn et al., 1993). OCCs may have a broad range of adverse effects on wildlife not only because of their acute toxicity but also because of their endocrine-disrupting activity (International Programme on Chemical Safety (IPCS), 2002; Matter et al., 1998).

Over the past twenty years, reptiles have received increasing attention as biomonitors of contaminant-induced endocrine disruption. Since many reptiles such as alligators and caimans are long-lived top predators, they are excellent models to study bioaccumulation and biomagnification of contaminants (Boggs et al., 2011; Stoker et al., 2011). In alligators, it has been demonstrated that OCP concentrations in maternal tissues and eggs are strongly correlated, allowing the mean egg burden to be used as a predictor of the OCP burden in maternal tissues (Rauschenberger et al., 2004).

Crocodilians are oviparous species characterized by laying elliptical eggs composed of a rigid calcareous shell. Eggshell layers assemble sequentially as the egg passes through the oviduct (Girling, 2002; Hincke et al., 2012; Lavelin et al., 2000; Palmer and Guillette, 1992). Since several steps in egg laying and eggshell formation are under hormonal control, exposure to EDCs such as OCCs may modify eggshell quality by disruption of hormonal signaling. The broad-snouted caiman (*Caiman latirostris*), which is widely distributed in South America, has the potential to accumulate OCCs. The eggshell of *C. latirostris*, is composed of two

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well-defined membranes: a calcareous, rigid and breakable external one, and a fibrous and flexible internal one (Paz et al., 1995). The hard membrane has pores that allow water and respiratory gases to be exchanged between the developing embryo and its external environment (Ar et al., 1974; Kern and Ferguson, 1997). Shell thickness and pore density greatly influence the success of embryonic development. In avian wildlife, reproductive failure due to eggshell thinning is one of the most serious ecotoxicological effects caused by dichlorodiphenyltrichloroethane (DDT) or its persistent metabolite dichlorodiphenyldichloroethylene (DDE) (reviewed in Fry, 1995). Interestingly, in laboratory studies, the evidence of eggshell thinning is specific regarding bird species and DDT metabolites. For example, ducks are sensitive to p,p'-DDE-induced eggshell thinning but domestic fowls are not. Indeed, o,p'-DDE, p,p'-DDT, o,p'-DDT, and p,p'-DDD do not cause eggshell thinning in ducks (Lundholm, 1997). Eggshell thinning in ducks has been correlated with p,p'-DDE inhibition of calcium ATPase in the shell gland (Lundholm, 1982). Moreover, embryonic exposure to o,p'-DDT alters carbonic anhydrase expression in the shell gland of domestic hens (Berg et al., 2004; Holm et al., 2006).

We have recently reported the OCC burden in *C. latirostris* eggs and the significant negative correlation between clutch size and OCC concentrations (Stoker et al., 2011). Here, we assessed the relationship between the burden of OCCs in *C. latirostris* eggs, eggshell features and hatchling survival.

2. Materials and methods

2.1. Study sample

All laboratory and field experiments were conducted according to the published guidelines for use of live amphibians and reptiles in field and laboratory research (American Society of Icthyologists and Herpetologists (ASIH), 2004), and in full compliance with the Institutional Animal Care and Use Committee of Universidad Nacional del Litoral (Santa Fe, Argentina).

The *C. latirostris* clutches evaluated in this report ($n=8$) were a subsample of those included in a study to assess the OCC egg burden and the relationship with parameters of reproductive performance (Stoker et al., 2011). Fig. S1 (Supplementary data) illustrates the habitats at nesting locations. To minimize OCC transfer from nest material or any post-laying environmental factors, eggs were harvested soon after they were laid and immediately transported to the laboratory (nesting was monitored by local residents). We opened one or two eggs per clutch in the field to stage embryos (Stoker et al., 2003). Only the nests with embryos at stages lower than twelve were transported to the laboratory. At the lab, egg mass was recorded to the nearest gram while the maximum egg lengths and widths were measured using a digital caliper (Fig. S2).

Two to four eggs from each clutch ($n=22$ eggs) were selected randomly to determine the eggshell features and the OCC egg burden (Wu et al., 2000; Sepúlveda et al., 2004; Rauschenberger et al., 2007). Embryos were removed and used for staging. Only eggs with embryos at stages 16–20 were used. The whole egg content (without the embryo) was stored at $-70\,°C$ until OCC analysis, in glass vials pre-cleaned with acetone/hexane. The chorioallantoic membranes were removed and then eggshells were washed for 15 min in distilled water, dehydrated in graded ethanols (70° and 96°, 15 min each), dried at room temperature and stored at $-20\,°C$ until analysis. The remaining eggs from each clutch were incubated in controlled conditions at $33\,°C$ or $30\,°C$ (temperatures at which 100 percent of males or females are produced, respectively) (Stoker et al., 2003). Upon hatching, neonates were identified using tags (style 1005-1, National Band and Tag Co., USA) and housed in controlled conditions to assess hatchling survival at ten days.

2.2. Detection of OCC residues

Homogenates from 22 eggs were analyzed for the presence of the following compounds: hexachlorobenzene (HCB), hexachlorocyclohexane isomers (α-HCH, β-HCH, and lindane), aldrin (ALD), oxychlordane (OCLD), α-chlordane (α-CLD), γ-chlordane (γ-CLD), heptachlor (HPT), heptachlor epoxide (HTX), dieldrin (DLD), endrin, mirex (MRX), methoxychlor (MTX), DDE (p,p'-DDE and o,p'-DDE), tetrachlorodiphenylethane (p,p'-TDE, o,p'-TDE), DDT (p,p'-DDT, o,p'-DDT), α-endosulfan (α-END), β-endosulfan (β-END), endosulfan sulfate (ENDSULF) and fourteen polychlorinated biphenyl congeners (PCBs) (BZ numbers 1, 5, 25, 28, 50, 52, 86, 101, 138, 153, 154, 180, 200 and 209).

Concentrations of OCCs were measured as described in Stoker et al. (2011). Briefly, for lipid extraction, thawed egg homogenates were dehydrated with anhydrous sodium sulfate (Merck, Germany) and extracted twice with n-hexane/acetone (4:1). The clear organic phase was separated and evaporated to dryness. The percentages of extractable lipids were gravimetrically determined in the residue (Mettler Toledo AB 204 scale). About 100 mg of lipids were dissolved in 0.3 mL of n-hexane. Then, 8 mL of acetonitrile (1 percent acetic acid) saturated in n-hexane was added, mixed by vortex and centrifuged. The organic phase was discarded. Then, 4 mL of n-hexane, 0.5 mL of a NaCl-saturated solution and 25 mL of water were added to the acetonitrile phase and mixed. The new organic phase generated was kept apart. The extraction step was repeated. The aqueous phase was discarded and the new organic phase was added to the former. The organic phase was evaporated to dryness and redissolved in 1 mL of n-hexane. The extract was transferred into a Solid Phase Extraction (SPE) cartridge containing Alumina (0.5 g), Florisil (1 g) and anhydrous Na_2SO_4 for the cleanup. Solid-phase extraction cleanup was performed using Supelclean LC-Alumina-N SPE tubes (Supelco, Bellefonte, PA, USA). The SPE cartridge was eluted with 10 mL of n-hexane and 5 mL of n-hexane/diethyl ether (85:15). The eluent was concentrated to 0.5 mL, and a second cleanup was performed using Supelclean Envi-Florisil SPE tubes (Supelco). The solvent was reduced to dryness with a nitrogen stream and redissolved in 1 mL of iso-octane.

Gas Chromatography (GC) analysis was performed with a Gas Liquid Chromatograph (Hewlett Packard Model 5890) 63Ni ECD. Pas 5 (25 m, 0.32 mm ID, film thickness 0.52 mm) and Pas 1701 (25 m, 0.32 mm ID, film thickness 0.25 mm) columns were used. Organochlorine pesticide standards Pestanal (Honeywell Riedel–de Haen Fine Chemicals, Seelze, Germany) and PCB congener standards (Ultra Scientific, North Kingstown, RI, USA) were used. *Gallus gallus* eggs (free of interfering compounds) were appropriate surrogates for caiman eggs for spiking purpose, thus were used as blank matrix. Linear regression curves adding standards in solvent and in blank matrix were done in order to evaluate matrix effects. Quantification was performed using calibration curves in blank matrix. Recovery assays were performed adding known concentrations of OCP and PCBs standards to the lipid extract of surrogate matrix blank. Aldrin was used as a reference standard to calculate relative retention times. No internal standards were added to samples in order to maintain sample integrity and original composition. All the solvents used were of pesticide-grade quality (Merck, Darmstadt, Germany). Recoveries for PCBs were in the range of 79–100 percent and for OCPs in the range of 84–100 percent. The Detection Limit (DL), based on three times the average Standard Deviation (SD) of the blank replicates, was 10 ng/g lipids for β-HCH, CLD, p,p'-DDT and ENDSULF, 2 ng/g for BZ 1, 5, 25, 86, 101, 138, 154, 180, 200 and 209, and 5 ng/g for the remaining OCPs and PCB congeners assessed. The Quantitation Limit (QL) was calculated as six times the average SD of blank for compounds with DLs between 5 and 10 ng/g and 7.5 times for those with DLs of 2 ng/g. The OCC values were calculated as the sum of OCP and PCB values. The presence and concentrations of OCCs were confirmed using a GC–MS system (VG Trio 2; VG Analytical, Manchester, UK) in randomly selected samples.

2.3. Evaluation of eggshell features

As mentioned above, eggshell samples came from eggs with embryos at stages 16–20. In a pilot study, eggshell thickness and porosity were evaluated in ten eggshell fragments from each egg region (equator and both poles). Samples came from three eggs of different nests. No differences in thickness or porosity were found between fragments from the equator and those from the poles (t test, $p < 0.05$). Therefore, to establish eggshell porosity and thickness, three eggshell fragments (one from the equator and one from each pole) were dissected. Values for each egg were pooled and values from eggs of the same clutch were averaged.

2.3.1. Eggshell pore analysis

Small fragments of eggshell (2–7 mm^2) were dissected using fine-tipped forceps and mounted to stubs under a stereoscopic microscope. Specimens were coated with a thin gold layer (with a Balzers sputter coater SCD 004). Images of the eggshell outer surface topography were acquired with a LEO 440i scanning electron microscope at $100 \times$ magnification. The number of open pores and the surface area of each eggshell fragment were established on scanning electron micrographs, using the image processing program Image J (NIH, Bethesda, USA), and expressed as pores/cm^2.

2.3.2. Eggshell thickness

Eggshell fragments (1 cm^2) were mounted individually on a sample holder and examined under the stereo-microscope Leica GZ6 (Leica Inc., Buffalo, NY, USA). Images were recorded by a SPOT color video camera (Diagnostic Instruments Inc., USA) attached to the microscope. Images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA). Spatial measurements were calibrated with reference rulers before every measurement.

2.4. Reproductive parameters

To assess caiman reproductive performance, clutch size (number of eggs in each nest), hatching success (number of eggs hatching successfully in each nest) and hatchling survival at ten days (percentage of survival at ten days of age in each nest) were recorded. Clutch size was established in the field during the collection process. The hatchability of eggs was calculated as the number of hatchlings divided by the total number of eggs during the entire incubation period.

2.5. Statistical analysis

Normal distribution of the data and homogeneity of variance (Levene test) were assessed previous to the use of parametric tests (Hogg et al., 2004). Since the distributions of OCC data were not normal in their original form, they were natural-log-transformed (ln) for statistical analyses. All values are expressed as clutch mean $\pm$ SEM. The OCC concentrations below the detection limit (BDL) were considered to be half of the detection limit. The OCC values below the quantitation limit (BQL) were considered to be half of the quantitation limit. t Test was used to compare thickness and porosity between eggshell fragments from equator and poles. Clutch mean values were used for correlations. Correlations were performed using Pearson analysis. Dixon's Q test was performed to identify outliers (Rorabacher, 1991). A value of $p < 0.05$ was accepted as significant.

3. Results and discussion

3.1. Clutch features and egg measurements

Data from each clutch are summarized in Table 1. Egg mass, length and width measurements recorded in the current study are similar to those previously reported for *C. latirostris* (Verdade, 2001; Stoker et al., 2003; Montini et al., 2006).

3.2. OCC residues in C. latirostris eggs

OCP residues were found in all clutches ($n=8$) (Table 2). The principal contributors to the OCP burden were the DDT family members (52.0 ± 10.5 ng/g lipid) and oxychlordane (17.8 ± 3.9 ng/g lipid). PCBs were present in 87.5 percent of the clutches (23.0 ± 4.0 ng/g lipid). The proportion and concentration of different OCCs are presented in Fig. S3. As previously observed, the OCC egg burden did not affect sex determination at the temperatures studied. Thus, all the eggs incubated at 30 °C resulted in female hatchlings and those incubated at 33 °C resulted in male hatchlings (Stoker et al., 2011). Moreover, consistent with the results reported by Beldoménico et al. (2007), the substantial nest effect seen in this experiment reflects the great variability existing in free ranging wildlife and contrasts with the homogeneity present within each brood.

3.2.1. Eggshell features, OCC burden, and hatchling survival

It has been shown that eggshell becomes thinner due to calcium uptake by the embryo, and that pore density increases at the end of the incubation period (Ferguson, 1982). In *C. latirostris*, incubation lasts from 65 to 75 days (Stoker, 2004). Therefore, to avoid any interference of sampling time on eggshell features, only eggs with embryos in the first quarter of the incubation period (embryonic stages 16–20) were used in the current study. Eggshell thickness and porosity data are summarized in Table 1. At this sampling period, the mean eggshell thickness was 400.9 ± 6.0 μm. Unlike that observed in birds, in which eggshell thinning by DDT metabolites has been reported as a major cause of reproductive impairment (Fry, 1995), we found no association between the total OCC egg burden and caiman eggshell thickness. The association was also absent when DDT family members or total pesticides were tested.

Fig. 1 shows scanning electron micrographs illustrating eggshell pore features. The number of open eggshell pores in the outer surface ranged from 8.9 to 39.3 pores/cm^2. In *Alligator mississippienssis*, different values of eggshell porosity were obtained when different regions of the eggshell were analyzed (Kern and Ferguson, 1997). We found no significant changes in the number of pores per square centimeter when samples from the equator and poles were evaluated (data not shown). This difference could be attributed to the limited sampling period (embryonic stages 16–20) used in our study. A significant inverse correlation was found between porosity and OCC burden (Pearson $r = 0.81$, $p = 0.01$) (Fig. 2). Pores allow water and respiratory gases to be exchanged between the developing embryo and its external environment (Ar et al., 1974; Kern and Ferguson, 1997). Therefore, a reduced porosity could compromise embryo and hatchling health. In birds, the effects of OCC exposures on embryos include, among others, mortality and reduced hatchability (Fry, 1995). Here

Table 2
Hatchling parameters and organochlorine compound concentrations in *C. latirostris* eggs.

Nest ID	Hatching success (percent)	Hatchling body mass at birth (g)	Hatchling survival at ten days of age (percent)	Σ OCPs (ng/g lipid)	Σ PCBs (ng/g lipid)
SF1	91.9	50.0 ± 0.3	91.9	61.9	11.6
SF2	85.2	35.0 ± 0.6	85.2	63.0	BDL
ER3	82.7	50.3 ± 0.4	79.3	28.9	BQL
ER4	76.0	43.7 ± 1.7	44.0	75.8	32.0
ER5	0.0	NA	0.0	189.0	44.5
CH4	91.2	43.9 ± 0.5	91.2	46.0	21.0
CH6	82.1	38.0 ± 1.0	82.1	31.0	13.0
CH7	86.5	49.5 ± 1.3	86.5	39.3	11.5

ID, identification according to nest location; NA, not available
SF, Santa Fe; ER, Entre Ríos: CH, Chaco.
ΣOCPs, sum of organochlorine pesticide residues; ΣPCBs, sum of polychlorinated biphenyls congener residues; BDL, below detection limit; BQL, below quantitation limit.

Table 1
Caiman latirostris nest locations, egg measurements and eggshell features.

Nest ID	Nest location	Clutch size	Egg mass (g)	Maximum egg length (mm)	Maximum egg width (mm)	Eggshell thickness (μm)	Eggshell porosity (pores/cm^2)
SF1	Santa Fe S30°15′ W60°50′	39	75.1 ± 2.4	69.3 ± 2.0	42.2 ± 1.7	419.3 ± 5.8	34.3 ± 2.76
SF2	Santa Fe S30°11′ W61°00′	35	60.2 ± 1.5	64.4 ± 2.1	39.9 ± 1.4	412.5 ± 1.2	32.4 ± 3.2
ER3	Entre Ríos S30°36′ W58°46′	33	72.0 ± 2.6	69.6 ± 1.0	41.2 ± 0.8	416.7 ± 6.9	39.3 ± 5.2
ER4	Entre Ríos S30°34′ W58°54′	28	65.5 ± 2.5	67.4 ± 1.4	39.9 ± 0.6	388.3 ± 7.8	8.9 ± 6.1
ER5	Entre Ríos S30°33′ W59°00′	18	64.3 ± 2.0	67.5 ± 2.2	39.8 ± 0.7	390.6 ± 6.7	8.6 ± 5.5
CH4	Chaco S26°48′ W59°03′	36	64.3 ± 1.7	64.1 ± 1.1	41.8 ± 0.9	414.0 ± 13.2	16.7 ± 10.5
CH6	Chaco S27°04′ W58°57′	30	59.9 ± 2.6	61.6 ± 2.6	41.5 ± 2.0	393.0 ± 7.9	27.1 ± 8.9
CH7	Chaco S26°47′ W59°15′	40	78.6 ± 4.4	69.4 ± 4.4	44.8 ± 2.4	372.0 ± 6.2	34.5 ± 12.35

ID, identification according to nest location; SF, Santa Fe; ER, Entre Ríos; CH, Chaco.
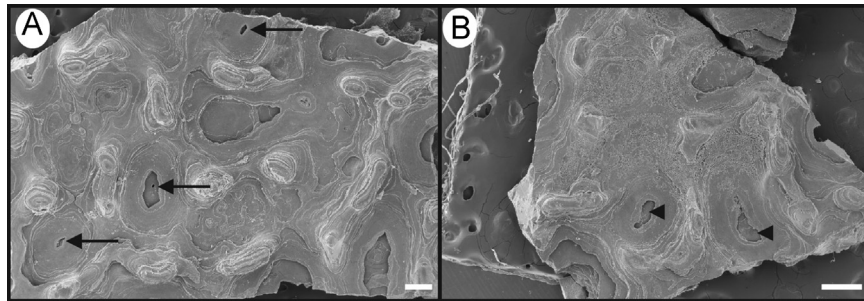All egg and eggshell measurements are expressed as Mean $\pm$ SEM.

Fig. 1. Representative scanning electron micrographs of *Caiman latirostris* eggshell pores. (A) arrows indicate open pores. (B) arrow heads indicate closed pores. Bars represent 200 and 300 μm, respectively.
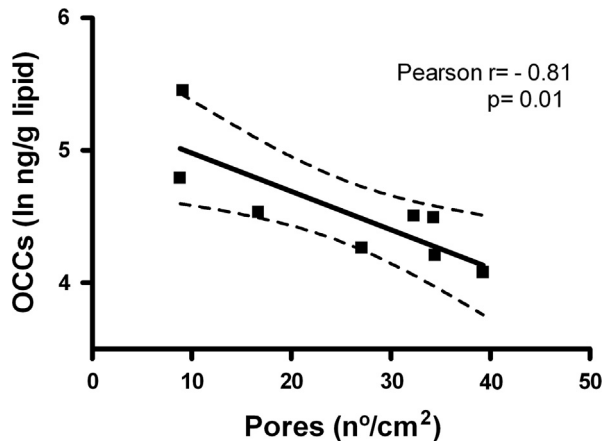

Fig. 2. Eggshell porosity in *C. latirostris* eggs is associated with organochlorine compounds (OCCs) burden. The higher the OCC burden, the smaller the number of pores.
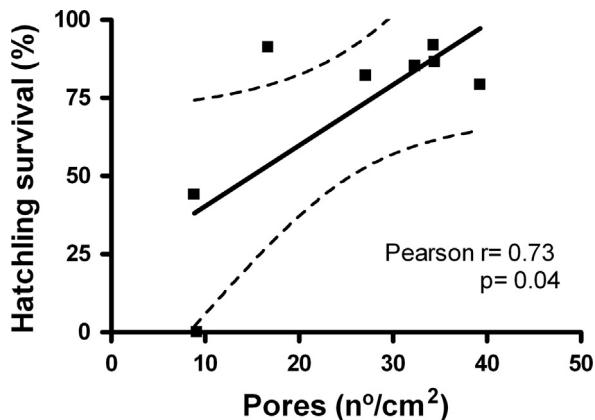

Fig. 3. Positive correlation between hatchling survival and eggshell porosity. Higher survival at ten days of age was observed in hatchlings born from clutches with higher eggshell porosity.

we found a positive correlation between hatchling survival and eggshell porosity (Pearson, $r = 0.73$, $p = 0.04$), showing a decrease in caiman survival as pore density decreased (Fig. 3). In American alligator embryos, chronic hypoxic incubation has been found to alter not only the embryonic growth pattern but also the morphological and physiological cardiovascular characteristics (Crossley and Altimiras, 2005). Such changes have also been observed in turtles and chickens incubated under hypoxic conditions (Metcalfe et al., 1984; Handrich and Girard, 1985; Kam, 1993) as well as in embryonic alligators with altered eggshell conductance (Deeming and Ferguson, 1989). Other factors such as the mother's nutrition and non-OCP contaminants could also influence clutch viability (Sepúlveda et al., 2004; Rauschenberger et al., 2009).

4. Conclusions

The present results demonstrate a significant inverse correlation between porosity and OCC egg burden. On the other hand, a decrease in caiman survival with decreased pore density was observed. Thus, we speculate that exposure to OCCs exerts a direct effect on the mother's oviductal functions, evidenced by decreased eggshell porosity, and an indirect effect on hatchling survival as a consequence of the chronic hypoxia experienced by eggs with a reduced pore density during their embryonic development. Our findings highlight another potential negative impact of current and past use of OCCs on wildlife species.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2013.08.022.

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