



Physical and chemical characteristics of fertile and infertile eggs of wild *Caiman latirostris*



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ARTICLE INFO

Keywords:

Broad-snouted caiman
Egg albumen
Fatty Acids
Protein
Lipid
Egg yolk

ABSTRACT

In oviparous species, physico-chemical characteristics of eggs are an indicator of maternal physiological traits and environmental conditions of the site where the females develop. In this study, we aim to (a) determine the physico-chemical characteristics of eggs from wild *Caiman latirostris* populations; (b) evaluate if fertile and infertile eggs differ in physico-chemical characteristics associated with such reproductive success; and (c) compare the present results with findings in other crocodylian species to identify characteristics that would be associated with reproductive biology and adaptations to their environments. Eggs were collected and identified, and different morphometric variables (percentage of components, and protein, total lipids and fatty acid composition) were determined. Fertile and infertile eggs did not differ in weight or component (eggshell, yolk and albumen) percentages of total egg weight. Fatty acid profiles of yolk differed between fertile and infertile eggs in C14:0, C16:1 and C17:1. However, the comparison of profiles among clutches showed differences in 12 fatty acids. We also observed differences in percentage of egg components respect to other crocodylian species, particularly of albumen, which had a lower proportion in *C. latirostris*. Our results suggest that egg infertility may not be related to maternal lipid investment. We propose using yolk of infertile eggs (which is similar to that of fertile eggs) as a non-invasive method to identify and evaluate the difference in FA composition of eggs for breedings female of varyns diets.

1. Introduction

In oviparous species, physico-chemical characteristics of eggs are an indicator of maternal traits and the environmental conditions in which females develop (Mousseau and Fox, 1998). In particular, age and body condition of females may affect physical characteristics and chemical composition. Reproductive experience in terms of both morphological and physiological maturity, is relate to age females, increasing their performance, at peak fertility, after which decline or senescence occurs (Williams and Christians, 2003; Bogdanova et al., 2006; Beamonte-Barrientos et al., 2010; Labaque et al., 2010). However, reproductive effort (e.g. quality and quantity of nutrients in the eggs) would be conditioned by females nutritional status, since resources are finite (Dunham et al., 1989).

External factors, such as environmental ones, nutrient availability, affect female diet and, therefore, has a direct influence on energy allocation to reproduction (White, 1991; Madsen and Shine, 1999;

Warner and Lovern, 2014). Hence, the nutritional status of reproductive females could be reflected in eggs composition variation (Verboven et al., 2003; Hargitai et al., 2009; Labaque et al., 2010, 2013), primarily the yolk fatty acid profile (Speake et al., 1998; Speake and Thompson, 1999; Surai et al., 1999; Surai and Sparks, 2001). This may affect the offspring phenotype and survival (Williams, 1994; Mousseau and Fox, 1998). Under this hypothesis, in years of favorable conditions (e.g., increased precipitation) and abundant nutrient resources (Simoncini et al., 2011), females are expected to be in better conditions than in unfavorable years, have a positive effect on their reproductive investment and, therefore, on the phenotype, adaptive capacity and offspring development (Marshall and Uller, 2007; Warner et al., 2015).

During egg production, females deposit nutrients in yolk and albumen, and minerals in egg shell. Yolk and albumen have been studied in numerous oviparous species and the relative proportions are used to classify eggs into altricial or precocial, with the former exhibiting a lower relative proportion of yolk and a higher one of albumen than the

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<https://doi.org/10.1016/j.aquaculture.2018.08.002>

Received 16 January 2018; Received in revised form 31 July 2018; Accepted 1 August 2018

Available online 02 August 2018

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latter. Non-avian reptiles have precocial eggs (Sotheland and Rahn, 1987; Nelson et al., 2010), a characteristic that reveals the evolutionary history of species (Deeming, 1991; Wallace et al., 2006). Egg components play different biological roles that are related to their principal contents. Yolk with its lipid content is the most important energy resource for embryo development, particularly fatty acids (FA), being the main nutrient component (Speake et al., 1998; Thompson et al., 1999; Thompson and Speake, 2004). FA from the lipid fraction of yolk provide information about maternal metabolism, diet variability, female investment, and those are essential for embryo development and for this reason, the FA are very useful for the study of reproductive biology of species. Albumen is the main source of water and proteins for embryo. Since 90% of albumen weight corresponds to water, it would be an important contributor to offspring biomass (Muramatsu et al., 1990; Moran Jr, 2007; Dzialowski et al., 2009). Vitamins and minerals associated with albumen proteins have bacteriostatic properties and/or protection functions in some oviparous species (White, 1991). The egg shell buffers changes in the external environment by allowing the exchange of water and gases that are crucial for embryo development (Ferguson, 1981, 1982; Ackerman et al., 1985; Grenard, 1991).

While there is abundant information about the relationship between egg chemical composition and its effect on offspring of some oviparous species, there are fewer studies on this subject for crocodylians (Jenkins, 1975; Burley et al., 1987; Noble et al., 1990, 1993; Noble, 1991). Several studies have addressed *Caiman latirostris* reproductive biology (Piña et al., 2003, 2007; Larriera et al., 2004, 2006; Simoncini et al., 2009); however, to date no study has been published on the physical and chemical characteristics of the eggs. *Caiman latirostris* is a valuable natural resource in Argentina and Brazil (Larriera, 1998; Verdade, 2001), therefore conservation and management programs have promoted research of its biology. In particular, Proyecto Yacaré is a program for the management of *C. latirostris* in Argentina that addresses the economic use of the resource and has promoted the conservation of the species and its habitat since 1990 (Larriera and Imhof, 2006). The program is based on egg collection from the wild, controlled incubation for captive breeding and release to the wild (Larriera et al., 2006). Further, studying the *C. latirostris* eggs characteristics will contribute to our knowledge on the species and provide tools for better decision making involving both captive and wild populations. Since through this studies we could in future to infer foraging patterns for evaluate the diet of the reproductive females of *C. latirostris* and the sources of acquisition of nutrients to be destined for reproduction. Moreover, in farm could to evaluate the effect of diet on reproductive performance (i.e. hatching success and phenotype of the individuals), and possible modifications of the diet for a productive improvement as well as the health of the broodstock and their offspring.

This work aims to (a) determine the physico-chemical characteristics of eggs from wild *C. latirostris* populations; (b) evaluate if fertile and infertile eggs differ in physico-chemical characteristics associated with reproductive success; and (c) compare these results with findings from other crocodylian species to identify characteristics associated with reproductive fitness and environments adaptations.

2. Materials and methods

We studied fertile and infertile eggs of *Caiman latirostris* from each clutch obtained from four nests (a total of 13 fertile and 9 infertile eggs) of a wild population in northern Santa Fe province, Argentina. We located nests in wild populations in the reproductive season, from early December 2013 (Simoncini et al., 2013); we collected the clutches shortly after oviposition, and transported to Proyecto Yacaré facilities in Santa Fe city (Santa Fe province, Argentina).

At Proyecto Yacaré, we determined the approximate time of oviposition (based Lungman et al. (2008); and we excluded in this experiment clutches with more than stage 5 embryo). The fertility state was ascertained for each eggs by examining the presence and size of the

opaque band. Selected eggs were stored at -18°C until further processing.

2.1. Physico-chemical analysis

Each egg ($N = 22$) was weighed with an electronic balance to the nearest ± 0.1 g and measured with caliper the long and short axes (± 0.1 mm). After opening the egg, we weighed the yolk, albumen and shell, and processed the yolk, according to Labaque et al. (2010). The lack of embryo confirmed infertility assessment (Manolis et al., 1987). The relative percentage of each component was calculated based on the total egg weight. Albumen was dehydrated at 60°C until constant weight, and the percentage of moisture was obtained based on the mass difference before and after dehydration.

We determined in 10 eggs (5 fertile and 5 infertile) nitrogen content (AOAC, 1980), protein values in dry albumen by multiplying total nitrogen by a conversion factor of 6.25 and lipid content for weight difference, by extraction from 10 g of dry yolk using chloroform and methanol (2:1) in a Soxhlet apparatus for 8 h (AOAC, 1980).

We processed and analyzed fatty acids in total eggs ($N = 22$, 13 fertile and 9 infertile eggs) by extraction of yolk lipids with chloroform and methanol (2:1) at room temperature. Lipids were subjected to alkaline saponification (1 N potassium hydroxide in methanol) and the unsaponifiable matter was extracted with n-hexane. The fatty acid methyl esters (FAMES) were obtained using 1 N sulphuric acid in methanol and analyzed by gas chromatography (GC) according to Labaque et al. (2010).

2.2. Data analyses

All data were analyzed using the statistical software INFOSTAT (Di Rienzo et al., 2016). Mean \pm standard deviation values are presented throughout the text. The physical and chemical characteristics of fertile and infertile eggs were analyzed using Generalized Linear Mixed Model (GLMM), with nest effect (or maternal effect) being used as random variable. Then we performed a General Linear Model (GLM) with egg fatty acid profile as dependent variable and nest as independent variable.

3. Results

Caiman latirostris eggs were ornamented (columnar microstructure; Fernández et al., 2013), oval and slightly ellipsoid, with a long axis length ranging from 6.14 to 7.60 cm (mean 6.72 ± 0.37 ; $N = 22$). The short axis length of eggs ranged from 3.95 to 4.47 cm (mean 4.23 ± 0.15 cm; $N = 22$). We did not find differences in egg weight or percentages of components (eggshell, yolk and albumen) between fertile and infertile eggs (Table 1). In both fertile and infertile eggs, total lipids accounted for approximately $22 \pm 0.25\%$ ($N = 10$) of total weight of yolk content, and protein content in albumen was $3.5 \pm 1.1\%$ ($N = 10$) of albumen weight (Table 2).

The analysis of total fatty acids (FA) composition in yolk of fertile and infertile eggs showed the presence of 15 fatty acids (Table 2). Considering both fertile and infertile eggs, the main FA recorded was oleic (C18:1), followed by palmitic (C16:0) and linoleic (C18:2), which accounted for 76%. We found differences in the profiles of three FA (Myristic C14:0; Palmitoleic C16:1; Heptadecanoic C17:1; $P < 0.05$) between fertile and infertile eggs, with higher values in fertile eggs. There were no differences in the proportions of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and SFA/UFA, SFA/MUFA and SFA/PUFA ($P > 0.05$; Table 2). However, the analysis of total FA composition among eggs showed differences in 73% ($N = 11$) of FA (C14:0, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C20:4, C20:5) and in 100% of the analyzed sums: SFA, UFA, MUFA, PUFA, SFA/UFA, SFA/MFA and SFA/PUFA ($P < 0.05$).

Table 1

Physical characteristics of fertile and infertile eggs of wild *Caiman latirostris* collected in Santa Fe (Argentina). Mean \pm standard deviation and range are indicated. No difference in physical characteristics between fertile and infertile eggs.

	Fertile eggs	Infertile eggs
Weight (g)	66.1 \pm 4.7 (range: 60.3–70.6)	66.7 \pm 5.4 (range: 60.5–71.7)
Eggshell (%) [*]	19.2 \pm 2.3 (range: 15.7–21.5)	19.5 \pm 2.6 (range: 14.2–21.5)
Albumen (%)	27.1 \pm 4.4 (range: 24.2–34.8)	28.5 \pm 5.1 (range: 24.4–35.1)
Albumen moisture (%)	94.9 \pm 2.3 (range: 89.5–96.4)	93.8 \pm 3.7 (range: 90.2–97.1)
Yolk (%)	53.9 \pm 2.6 (range: 49.5–56.3)	52.1 \pm 3.7 (range: 47.4–56.9)
Yolk moisture (%)	46.16 \pm 2.10 (range: 43.1–48.7)	46.38 \pm 3.25 (range: 42.3–51.2)

* Values of eggshell include shell membrane.

Table 2

Chemical composition of fertile and infertile eggs of wild *Caiman latirostris* in Santa Fe (Argentina).

Components	Fertile egg	Infertile egg
Yolk lipids (%)	22.1 \pm 0.12	22.2 \pm 0.30
Albumen protein (%)	3 \pm 0.24	4.2 \pm 2.40
Fatty acids		
Saturated		
C 14:0 Myristic [*]	0.66 \pm 0.20	0.61 \pm 0.20
C 15:0 Pentadecylic	0.55 \pm 0.20	0.49 \pm 0.13
C 16:0 Palmitic	23.65 \pm 1.31	23.06 \pm 1.71
C 17:0 Margaric	1.40 \pm 0.48	1.50 \pm 0.41
C 18:0 Stearic	7.02 \pm 0.52	7.11 \pm 0.35
Unsaturated		
Monounsaturated		
C 16:1 Palmitoleic [*]	5.79 \pm 1.16	5.65 \pm 1.23
C 17:1 Heptadecanoic [*]	1.45 \pm 0.45	1.32 \pm 0.37
C 18:1 Oleic	44.46 \pm 4.50	44.85 \pm 3.03
Polyunsaturated		
C 18:2 Linoleic	8.65 \pm 1.24	8.96 \pm 1.35
C 18:3 Linolenic	0.13 \pm 0.08	0.17 \pm 0.16
C 20:4 Arachidonic	3.35 \pm 0.46	3.29 \pm 0.52
C 20:5 Eicosapentaenoic	0.61 \pm 0.22	0.66 \pm 0.20
C22:4 Docosatetraenoic	0.12 \pm 0.16	0.11 \pm 0.17
C 22:5 Docosapentaenoic	0.50 \pm 0.19	0.64 \pm 0.43
C 22:6 Docosahexaenoic	1.36 \pm 0.48	1.58 \pm 0.65
Saturated (SFA)	33.31 \pm 1.90	32.58 \pm 1.58
Unsaturated (UFA)	66.68 \pm 1.90	67.41 \pm 1.58
Monounsaturated (MUFA)	51.95 \pm 1.07	51.82 \pm 1.74
Polyunsaturated (PUFA)	14.73 \pm 1.47	15.59 \pm 1.73
SFA/UFA	0.50 \pm 0.04	0.48 \pm 0.03
SFA/MUFA	0.64 \pm 0.04	0.62 \pm 0.04
SFA/PUFA	2.28 \pm 0.32	2.11 \pm 0.28

* Significant differences in yolk between fertile and infertile eggs at $P < 0.05$. Mean \pm standard deviation and range are indicated.

4. Discussion

This is the first report of physico-chemical composition of *C. latirostris* eggs collected from the wild. While we detected differences in the profile myristic C14:0, palmitoleic C16:1, heptadecanoic C17:1 acids between fertile and infertile eggs. This differences may not have influence on egg fertility, indeed, fertile and infertile eggs had similar values of total SFA, and MUFA; therefore, egg infertility would not be related to maternal lipid investment. The proportion of fertile eggs in the clutch of these reptiles may be affected by other factors, such as the age of females; accordingly, it has been suggested that in *Alligator mississippiensis* senescent or very young females would have a higher number of infertile eggs (Ferguson, 1985). Moreover, egg fertility can

Table 3

Percentage \pm standard deviation of eggshell, albumen, yolk and weight of eggs for six species of crocodylians.

Species	% Eggshell	% Albumen	% Yolk
<i>Caiman latirostris</i>	19.2 \pm 2.3	27.8 \pm 4.4	53.0 \pm 3.1
<i>Crocodylus intermedius</i> ¹	13.9 \pm 1.9	42 \pm 0.8	43.8 \pm 1.3
<i>Alligator mississippiensis</i> ²	12 \pm 0.3	46.5	40.6 \pm 1
<i>Alligator mississippiensis</i> ³	–	44	–
<i>Crocodylus novaeguineae</i> ⁴	14 \pm 0.9	–	–
<i>Crocodylus johnstoni</i> ⁵	11.9 \pm 0.3	42.1 \pm 0.8	46 \pm 0.8
<i>Crocodylus porosus</i> ⁵	12 \pm 0.4	45.7 \pm 0.8	42.4 \pm 0.8

¹Morales Arango et al., 2007; ²Ferguson, 1985; ³Tracy and Snell, 1985; ⁴Calculated from Jenkins, 1975 cited by ⁵Manolis et al., 1987.

also be affected by sperm deficiencies in males (Salem Jr., 1989; Noble et al., 1993; Lance et al., 2001), and even by male exposure to contaminants (Rauschenberger et al., 2007), which was not evaluated in this study. Thus, our results regarding similar composition of FA in yolk of fertile and infertile eggs suggest that fertilization is independent of yolk composition and, therefore, yolks of infertile eggs could be used as a noninvasive method, applying different techniques (fatty acids, isotopes, among others) for the study of the diet, maternal investment and nutritional status of the species, without affecting eggs with possible embryonic development.

The percentages of eggshell and yolk in eggs of *C. latirostris* were 5% and 7% higher, respectively, than values reported for other species, such as *Crocodylus johnstoni*, *Crocodylus porosus*, *Crocodylus novaeguineae*, *Crocodylus intermedius* and *Alligator mississippiensis* (Manolis et al., 1987; Morales Arango et al., 2007), whereas albumen was 14% lower than values reported for other crocodylians species (Manolis et al., 1987; Morales Arango et al., 2007; Table 3). The lower percentage of albumen should be further analyzed, since this component plays important roles, such as making essential proteins available to the embryo and providing chemical protection; it also has and antifungal and antimicrobial activities (White, 1991; Thompson and Speake, 2003; Willems et al., 2014) and avoids embryo dehydration during development (Manolis et al., 1987; Nelson et al., 2010).

Values of total lipids in yolk and protein content in albumen are similar to those reported for other wild crocodylian species (Manolis et al., 1987; Noble et al., 1993). Nelson et al. (2010) suggested that yolks of crocodile eggs would contribute the highest proportion of solids (protein, trace minerals, and vitamins) for embryo development, whereas albumen contains a low percentage of solids; therefore, the authors suggest that the main role of albumen would be that of water reservoir. Hence, the differences in albumen percentage of *C. latirostris* eggs respect other crocodylian species, would indicate that our study species has specific evolutionary traits, which would be reflected in a lower proportion of albumen in the egg than in other species. Accordingly, this lower albumen proportion in eggs of *C. latirostris* would be a result of the adaptation to an environment of high moisture levels and water availability (Tracy and Snell, 1985). In agreement with this idea of change in response to the environment, a recent study reported the functionality of rugged structures in eggshells (eggs ornamentation; Fernández et al., 2013) of *C. latirostris*, these structures produced air bubbles when submerged, thus providing an oxygen reservoir during the brief time when the nest is flooded (Cedillo-Leal et al., 2017). This provides the embryo with a greater tolerance to nests exposed to floods, mitigating the embryo loss and/or damage caused by early inundation evidencing physical characteristics of the egg that would respond to high water availability in the environment. Therefore, albumen percentage would be the minimum water invested by the female for embryogenesis, whereas the remaining need for water would be met by the incubation environment.

Differences in component percentages of *C. latirostris* eggs might be also attributed to the smaller size of eggs than that of other species

(Webb et al., 1987; Ardila et al., 1999; Morales Arango et al., 2007). There is evidence of intraspecific variability in percentage of egg components within a clutch depending on egg size, where albumen mass increases more rapidly with egg mass than does yolk mass (Dzialowski et al., 2009; Nelson et al., 2010). Hence, a smaller egg size than that of other species might condition albumen percentage.

Regarding fatty acid composition in the yolk lipid fraction, some components were not detected in some clutches and in other clutches in trace amounts, such as linolenic (C18:3) and docosatetraenoic (C22:4) fatty acids. This is an interesting observation, since differences in some FA in eggs from different clutches (C18:2; C20:4; C20:5) coincide with that observed in other avian oviparous species (Surai and Sparks, 2001; Labaque, 2006; Polito et al., 2012) and non-avian reptiles (Lovern and Adams, 2008; Craven et al., 2008). Particularly within the egg PUFA profiles, some FA (essential fatty acids) cannot be synthesized by vertebrates and can only be acquired through the diet (Miyazaki and Ntambi, 2008). Therefore, differences in some FA in the yolk can be related to the quality of the diet consumed by females that produce the eggs.

Respect to the diet, as reported for the species (Borteiro, 2005), most frequent prey found in the diet are insects, mollusks, vertebrates (amphibians, fish and reptiles) and crustaceans, to a lesser extent arachnid. Based on previous studies of other reptile species, we can infer that these food items are potential sources of certain essential fatty acids. Records indicate that C20:4 and C22:6 are respectively the characteristic PUFA in carnivorous and piscivorous eggs due to their predominance in particular diets (Speake and Thompson, 1999). It is well established that C18:2n-6 and/or C18:3n-3 are the predominant PUFA of terrestrial insects (Stanley-Samuelson and Dadd, 1983; Stanley-Samuelson et al., 1988). Thus, the high ratio of C22:6 in *Alligator mississippiensis* reflects consumption of aquatic organisms (Noble et al., 1993), and in other reptiles the prevalence of C20:4n-6 in buds is consistent with a diet of mammalian prey (Speake et al., 2003).

It is important to pay attention to PUFA in the diets of reproductive females, as they are important for maternal investment, since PUFA are essential during embryonic development (Speake et al., 1994, 1998; Navarro et al., 2001; Labaque et al., 2013). The need for PUFA during embryonic development has been explained by their role in a number of metabolic characteristics that allow both growth and tissue function (e.g., brain and retina development; Speake and Thompson, 1999, 2000). In captive females, the fertility and hatchability of eggs can be increased by paying special attention to the levels of PUFA and their active protection against oxidation in the diets of breeding animals (Noble et al., 1993).

It is important to note that previous studies in reptiles suggest that PUFA would be influenced not only by the diet, but also by the interaction between FA incorporated by the diet, maternal metabolism and environmental conditions (Noble et al., 1993; Craven et al., 2008; Warner and Lovern, 2014). According to the results obtained in the present study, future studies of these questions will be developed using infertile eggs as a non-invasive evaluation tool for this species.

5. Conclusions

Our results shown that FA profiles and the amount of lipid in egg yolks did not differ between fertile and infertile eggs and suggest that fertilization is independent of yolk composition. Therefore, it is possible to use infertile eggs as a potentially noninvasive method for identifying and evaluating FA composition in a clutch of eggs, that would allow infer differences in diets of breeding females and provide an important tool to evaluate the status of wild populations of *C. latirostris* in respect to the percentage values of egg components of species, differences suggest specific evolutionary traits.

Future studies should assess the maternal effect of *C. latirostris* females on nutrient transfer to the yolk and their reproductive success, as well as the interaction with climatic variables, such as precipitation and

temperature, which would condition resource availability and female capacity to invest in reproduction. This information would support decision making for conservation and management and may also be extrapolated to farm or captive-bred populations with the aim to propose management strategies via egg evaluation and diet improvement.

Acknowledgments

We thank other members of Proyecto Yacaré. This study was supported by Proyecto Yacaré, Yacaré Santafesinos (Gob. Prov. Santa Fe/MUPCN), PFIP 2008; and PICT 2014 N2138 (to C. Piña), PICT 2014 N2212 (to M. Simoncini), and CAI+D 2017 (to M. González and M. Simoncini). This is the publication 102 from Proyecto Yacaré.

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