Subject: MS 13-54, "Fungal diversity on broad-snouted caiman (*Caiman latirostris*) eggs, and their effects on hatchlings"

Decision: Accept

Dear Dr. Nuñez Otaño,

I finally got the time to once again look at the revised 13-54 (apologies for the slight delay!). All formal problems have been dealt with , and I have accepted it for the Herpetological Journal.

The paper will likely appear in issue 4/2014 (scheduled for October). We will send you the page proofs in due course, please contact the Managing Editor (Chris Barratt, c.d.barratt@gmail.com) if you have any further questions regarding the production of your paper. From 2014 onward we can also offer open access free of charge for BHS members, and at a publication fee of 97.- UKP for non-members.

Once again thanks a lot for submitting this interesting paper to the Herpetological Journal.

With best wishes,

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Fungal diversity on broad-snouted caiman (*Caiman latirostris*) eggs, and their effects on hatchlings

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Running title: Fungal isolation on *Caiman latirostris* eggs

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ABSTRACT

Studies describing and identifying mycobiota affecting the eggs of wild reptiles are rare, despite the potential importance of mycoses for the survival and performance of individuals and populations. The aim of this study was to identify the fungal species on eggshell and eggshell membranes of *C. latirostris* and to discover potential compositional changes between these two substrates. Twenty-four species of fungi were isolated from eggshells and 17 species were isolated from membranes; 10 species were shared between both substrates. Saprophytic fungi comprised 64.1% of eggshell and 59.4% of eggshell membranes mycobiota, respectively. Potentially pathogenic fungi occurred more frequently on the eggshell membrane (71.4%). From pathogenic assays we cannot conclude that fungi like *Aspergillus fumigatus* and *Fusarium oxysporum* have a negative effect on hatching success, weight and snout-vent length of *C. latirostris* hatchlings.

Keywords: Fungal diversity, egg colonization, trans-shell infection, *Caiman latirostris*.

INTRODUCTION

The interactions among fungi and animals can take a multitude of forms, and have significant effects on ecosystems. Many pathogenic infections are not fatal but can influence the fitness of the host, which in turn can have consequences on the species' population dynamics (Dighton, 2003). Fungi-host relationships have been studied in eggs of sea turtles (Phillot et al., 2004; Elshafie et al., 2007; Güçlü et al., 2010), amphibians (e.g. Baláž et al., 2012; Gower et al., 2012), lizards (Moreira Lopes & Barata, 2005), snakes (Paré et al., 2003) and crocodiles (Buenviaje et al., 1994; Thomas et al., 2002). Oviparous female reptiles often deposit clutches in close

proximity to each other (Pianka & Vitt, 2003). If a few non-viable eggs are laid within a clutch, the nest success may be jeopardized because these eggs could promote colonization by fungi which are hemibiotrophs (HB) also for viable eggs (Cooke & Whipps, 1993; Phillot & Parmenter, 2001; Robinson et al., 2003). Microfungi penetrate into the eggshell through pores or eggshell cracks (Paz et al., 1995; Williams et al., 2000), and some fungi can be transmitted to the egg during oviposition (Phillot et al., 2002; Nuñez Otaño et al., 2013). After oviposition, the shell can acquire microbial contaminants from all surfaces with which it makes contact (De Reu et al., 2006). The warm and moist microenvironment and the presence of organic matter at reptile nesting sites are beneficial for the growth of soil fungi that might decrease hatching success, whether decomposing the eggshell and/or secreting mycotoxine that adversely affects the developing embryos (Patino-Martinez et al., 2012).

The ranching program "Proyecto Yacaré" allows collecting caiman eggs from natural nests, with an attempt to improve hatching success through incubation under artificial conditions. Failed eggs can provide a nutrient source for common soil fungi, and can enhance the progressive spread of hyphae to adjacent eggs during incubation (Phillot & Parmenter, 2001; Moreira Lopes & Barata, 2005). Phillot & Parmenter (2001) showed that in extreme situations the entire egg mass was enveloped by hyphae and resulted in a reduced hatching success. Several studies strongly suggest that molds like *A. fumigates* and *F. oxysporum*, which cause human disease, may not be typical obligate aerobes but rather are facultative anaerobes (Grahl et al., 2012). The aim of this study was to provide the first description of fungal diversity highlighting pathogenic fungi on eggshell and eggshell membrane of *C. latirostris*. Furthermore, we wanted to test the effects of pathogen fungi like *Aspergillus fumigatus* and *Fusarium oxysporum* on hatching success, weight and snout-vent length of hatchlings.

MATERIALS AND METHODS

Eggs were collected during the reproductive season (from December of 2010 to January of 2011) from nests of levees (n = 2), bushes (n = 1) and floating vegetation (n = 3), in the north of Santa Fe province, Argentina. Eggs were collected and stored at 4 – 7 °C in sterile polyethylene. Mycobiota on the eggshells (ES) and eggshell membranes (ESM) were sampled as described by Mueller et al. (2004). A total of 300 particles (150 particles for eggshells and 150 particles for egg membranes) were seeded on Malt Extract Agar (MEA 20%; 20 g Malt; 1g peptone; 20 g Agar; 20 g Glucose). The agar contained the antibiotics streptomycin (5 mg ml^{-1}) and chloramphenicol (2.5 mg ml^{-1}) to minimize bacterial growth. Plates were incubated at ambient temperature and natural photoperiods, and were examined macroscopically and microscopically (Leitz-Dialux 20 EB) after a minimum of seven days. Identification was achieved by taxonomic processes such as direct comparison of specimens and by the use of keys, descriptions and illustrations. We consider one CFU (Colony Forming Unit) as an individual. Cultures were labeled as LPSC 2101 through LPSC 2138 and deposited in the culture collection of the Institute Carlos Spegazzini (La Plata, Buenos Aires, Argentina). Nutritional modes were used as a mean of delimitating econutritional categories of behavior according to whether fungi were biotrophic-pathogen or biotrophic-saprotrophs (Cooke & Whipps, 1993).

After fungi identification, collection effort curves of ES and ESM were made with EstimateS 8.2.0 (Colwell, 2009) to evaluate sampling effort using abundance data available and CHAO 1 as richness estimator. Diversity analyses of each substrate was made with PAST (Hammer et al., 1999; PAleontolical STatistics v. 1.90), considering Richness (S), Abundance (N), also Equitability (J´), Dominance (D) and Shannon-Wiener index (H´).

Fungal bioassays

The eggs of 3 nest (n = 68) were harvested soon after laying (from December of 2011 to January of 2012), incubated at 31 ± 0.5 °C (the mean temperatures of nests in the field, Piña, 2002), and buried in moist sterile vermiculite (four parts sterile distilled water to three parts sterile vermiculite by mass). Opaque patch development was a determinant to separate fertile from infertile eggs (Iungman et al., 2008). Temperature was monitored by a HOBOTM Data Logger (Onset Computer Corporation, Pocasset, MA). Air humidity was assumed to be high (90–100% relative humidity) and nests were kept humid with sterile water from a clean spray bottle. Prior to the experiment, incubators were cleaned, and sterile conditions were maintained during the transport, handling and incubation of eggs (procedures following Phillot, 2002). Eggs were placed in a single layer to avoid temperature and humidity gradients (Ewert & Nelson, 2003).

The experiment was divided into control (C: sterile distilled water), treatment 1 (T1: 10^7 conidial/ml) and treatment 2 (T2: 10^{11} conidial/ml) for each species of fungus. Five eggs for each treatment were randomly selected and sprayed with 600 ul of water and conidial suspensions at the beginning of incubation before being placed into six plastic containers (three treatments with two replicates each) for each fungi involved in the experiment (n_{total} = 30 eggs for every species of fungus). The containers were opened every four days to allow gas exchange at the onset of incubation, and daily whenever opaque egg patch have covered almost the entire egg.

Fungal isolates were grown on agar in 9 cm petri dishes for one week at 30 °C. *Aspergillus fumigatus* was isolated from eggshell membranes samples and *Fusarium oxysporum* was isolated from nest material (Nuñez Otaño, 2013). For fungal bioassays, conidia were harvested with disposable cell scrapers (Fisherbrand) and placed in test tubes containing 0.01% (v/v) Tween 80 (Merck). Suspensions were vortexed for 2 minutes, filtered through four layers of

sterile muslin, and adjusted to 10^7 and 10^{11} conidia/ml after cell enumeration in a Neubauer hemocytometer for both fungal species (Pelizza et al., 2012). The viability of the conidia from all isolates used in the tests was determined after 24 h (following Lane et al., 1991).

During incubation, eggs were controlled through ovoscopy and by eye for eggshell sanity. Failed eggs were removed. After hatching, hatchlings were weighed (g) and measured (SVL in mm). Weight and SVL were analyzed using a non-parametric one-way analysis of variance (Kruskal Wallis); tests were run for each treatment x species of fungi. For *Fusarium oxysporum* assays, one nest was not considered due to the lack of data.

RESULTS

We cultured 300 particles of egg shells and egg shell membranes from six eggs and isolated 129 CFUs of 31 fungal species. In general, eggshells had a higher abundance and richness of fungi (71.3% of CFUs and 77.4% of total species, Appendix 1). Seven species were exclusively found in egg shell membranes; 71.4% of species were biotrophic-pathogens and 28.6% were biotrophic-saprotrophs (Table 1). Ten species were isolated from both substrates (40% were saprotrophs and 30% pathogenic fungi, Appendix 1), and only *Syncephalastrum racemosum*, *Penicillium turolense* and *Dematiaceous mycelia sterilia* were present on both substrates of the same eggs; *S. racemosum* was more persistent on the egg shell (23 CFUs); the other two species occurred less frequently. Other common species were recorded either on the eggshell or the eggshell membranes of different eggs (Appendix 1). Diversity analyses showed low values of dominance (0.11 – 0.097) in accordance with high values for equitability (0.83 – 0.91). The Shannon – Wiener index was similar between egg shells (H´= 2.641) and egg shell membranes (H´= 2.578, Shannon diversity t-Test; $p = 0.4$).

The accumulation curves for egg shells revealed that 72.7% of the expected species (33 ± 7.62) (CHAO 1 ± SD) were sampled (24 ± 2.46, S_{obs} ± S_{obs} SD, Fig. 1). Of the 24 fungi species isolated from egg shells, 62.5% were biotrophic saprotrophs, 25% were biotrophic pathogens, 8.3% were non-sporulating and 4.2% (1 out of 24) were yeast. Richness values ranged between five and seven species per egg, and fourteen species only occurred on egg shells (Table 1). In total, 57.1% of species were biotrophic pathogens and 42.8% were biotrophic saprotrophs. The most abundant nutritional group was represented by biotrophic saprotrophs (64.1%), followed by biotrophic pathogens (25%), yeast and non-sporulating fungi (5.4% each). *Syncephalastrum racemosum* and *Rhizopus stolonifer* showed elevated abundance values and together represent 40.2% of the total fungi abundance for egg shells (Appendix 1).

For egg shell membranes, the accumulation curves revealed that 87.8% of the species expected (19 ± 3.18, CHAO 1 ± SD) were sampled (17 ±2.11, S_{obs} ± S_{obs} SD, Fig. 2). A total of 17 fungal species were isolated from eggshell membranes; 52.9% were biotrophic saprotrophs, 29.4% were biotrophic pathogen, 11.8% were non-sporulating fungi and 5.9% were yeast (Appendix 1). Species richness was highest at S = 7 for one egg, and ranged between 1 and 4 species for all other samples. The total abundance was 37 CFUs; 59.4% were biotrophic saprotrophs, 32.4% were biotrophic pathogens, 5.4% were non-sporulating fungi and 2.7% (1 CFU of 37) were yeast. *Cladosporium cladosporioides* (18.9%) and *Syncephalastrum racemosum* (16.2%) had high abundance values, followed by seven species with intermediate abundances (5.4% - 10.8%).

Hatching success from *Aspergillus fumigatus* assays was 100% for controls, 90% for treatment 1 and 80% for treatment 2. Bioassays with *Fusarium oxysporum* resulted in 60% for controls, 100% for treatment 1 and 80% for treatment 2. There was not negative trend in hatching

success when conidial concentrations increased. Hatchling weight differed between groups ($p < 0.05$ for both *A. fumigatus* and *F. oxysporum* essays, however without a trend depending on treatment. There was no negative effect on hatchling SVL (*A. fumigatus*: $p = 0.74$; *F. oxysporum*: $p = 0.58$).

DISCUSSION

It is possible that soil-growing fungi contaminated the eggs when they were laid and/or during passage through the cloaca (Phillot et al., 2002, 2006; Elshafie et al., 2007; Nuñez Otaño et al., 2013). Biotrophic-saprotroph fungi were abundant on egg shells and egg membranes, in line with the fact that they for example represent an estimated of 78 – 90% of the total microbial biomass of descomposing grassland (Frankland, 1982). Richness and abundance values for fungi were higher on egg shells than egg shell membranes, probably as a result of egg shell structure. The egg shell in Crocodylia is white, rough, and has internal fibrous membranes, pores, craters and pore plugs during the first weeks of incubation. The latter consists of bacteria, fungi, remnants of nesting material and oviductal secretions (Goodwin & Marion, 1978; Ferguson, 1982; Paz et al., 1995; Kern & Ferguson, 1997), serving as a barrier between the external environment and egg contents (Berrang et al., 1999). According to Fernández et al. (2013), the egg of *C. latirostris* contains less than 0.03 pores/mm^2, and it is possible that the egg shell serves as a barrier for fungi due to the low proportion of pores connected to the egg membrane. However, fungal colonisation of the egg membrane could take place through percolation of conidia through pores in egg shells, and through cracks in the shell (Ferguson, 1982).

Species of the genus *Aspergillus*, *Penicillium* and *Fusarium* commonly isolated from both substrates are known reptilian pathogens (Jacobson et al., 2000; Huchzermeyer, 2003; Mitchell & Tully, 2009). *Aspergillus* spp. can grow at different temperatures and substrates, and produce

aflatoxins as secondary metabolite. *Aspergillus niger* has been isolated from *Chelonias mydas* eggs (Elshafie et al., 2007) and in the cloaca of the *C. latirostris* (Nuñez Otaño et al., 2013). *Aspergillus fumigatus* has also been isolated from partially decomposed vegetation from in nests of *C. crocodylus fuscus* (Tansey, 1973), and can have lethal effects on eggs, hatchlings and young caimans (Palacios & Sick, 2004). It was also isolated from lung samples of captive *Alligator mississippiensis* (Jasmin et al., 1968) and in neonate *Crocodylus porosus* skin lesions (Buenviaje et al., 1994). *Aspergillus flavus* found on egg shells is a pathogen with a worldwide distribution, however found mainly in tropical and subtropical regions (Domsch et al., 1993).

Penicillium, found on egg shells and egg shell membranes in the present study, is common in natural environments (Samarajewa, 1991); some species produce secondary metabolites which can have physiological effects on hosts (Pitt & Hocking, 1997). *Fusarium* is a genus with a worldwide distribution and encompasses saprotrophic, biotrophic-pathogenic or endophytic fungi (Pier et al., 1980). Several *Fusarium* species produce mycotoxins (secondary metabolites include fumonisins) and it is known to cross egg shell membranes of reptiles (Hibberd & Harrower, 1993; Cabanes et al., 1997), resulting in egg loss and low hatching success (Moreira Lopes & Barata, 2005; Phillot et al., 2006). *Fusarium oxysporum* affects hatchlings and young turtles (Jacobson et al., 2000; Phillot & Parmenter, 2001; Elshafie et al., 2007; Güclü et al., 2010; Sarmiento-Ramirez et al., 2010; Patino-Martinez et al., 2012), and is responsible for lesions on eggshell membranes in infertile eggs of *Crocodilus porosus* (Schumacher & Cardeilhac, 1990). Infected incubated eggs however do not lead to reduced hatching success in several species studied (Larriera et al., 2006; Patino-Martinez et al., 2012).

Given that 19% and 32% of fungal species identified on eggs, respectively, were also found in female cloaca and nest substrate (see also Nuñez Otaño et al., 2013), our result supports the hypothesis that the environment serves a as propagule for fungal infections on egg shells,

given that microenvironmental conditions such as warm temperature and high humidity are in favour of fungal growth (Sarmiento-Ramirez et al., 2010). The presence of conidia on egg shells not necessarily leads to the loss of eggs under natural conditions (personal observation).

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APPENDIX

Mycobiota isolated from **ES** (Egg shells) and **ESM** (Egg shell membranes) of *C. latirostris* (Number of CFUs per fungal species in every column*).* Nesting habitat: B (Bushes), FV (Floating vegetation) and L (levee).

Econutritional Category	Species and Authority	ES							ESM						
		B	FV			L		N	B	FV			L		N
		1	1	2	3	1	2		1	1	2	3	1	2	
Biotrophic-Pathogen	*Alternaria alternata* (Fr.) Keissl. 1912	1				2		3							
	Aspergillus flavus Link 1809	2						2							
	Aspergillus fumigatus Fresen. 1863								1				1		2
	Aspergillus niger Tiegh. 1867												2		2
	Aspergillus sp. P. Micheli ex Link 1809								2						2
	Curvularia lunata (Wakker) Boedijn 1933			2	5			7				4			4
	Fusarium sacchari (E.J. Butler & Hafiz Khan) W. Gams 1971			1	4			5							
	Fusarium sp. Link 1809		2	2			2	6				1	1		2
	Fusarium sp2. Link 1809				1		1	2							
	Fusarium sp3. Link 1809				1			1							
	Acremonium butyri (J.F.H. Beyma) W. Gams 1971											1			1
Biotrophic-Saprotrophs	*Acremonium strictum* W. Gams 1971				1			1							

	Alternaria tenuissima (Kunze) Wiltshire 1933					2	2									
	Arthrographis sp. G. Cochet ex Sigler & J.W. Carmich. 1976.		1				1									
	Cladosporium cladosporioides (Fresen.) G.A. de Vries 1952	1			1	1	3		1	1		4	1	7		
	Cladosporium herbarum (Pers.) Link 1816		1				1					2		2		
	Cladosporium sphaerospermum Penz. 1882	1					1									
	Eurotium herbariorum (F.H. Wigg.) Link 1809								1					1		
	Penicillium echinulatum E. Dale 1923	1					1									
	Penicillium funiculosum Thom 1910		1				1									
	Penicillium sp. Link 1809	1					1									
	Penicillium turolense C. Ramírez & A.T. Martínez 1981				1		1					1		1		
	Penicillium verrucosum Dierckx 1901		3			2	5						2	2		
	Phialocephala fusca W.B. Kendr. 1963												1	1		
	Rhizopus stolonifer (Ehrenb.) Vuill. 1902			14			14									
	Scopulariopsis acremonium (Delacr.) Vuill. 1911								1					1		
	Syncephalastrum racemosum Cohn ex J. Schröt. 1886	21	2				23	5	1					6		
	Trichoderma harzianum Rifai 1969			1			1									
Non Sporulating	*Dematiaceous mycelia sterilia*					2	2						1	1		

	Hialine mycelia sterilia	1	1	1	3			1		1	
Yeast	*Rodhotorulla sp.* Harrison 1927			5	5				1	1	
	Abundance (CFU)				**92**					**37**	
	Richness				**24**					**17**	

363 FIGURE CAPTIONS

364 Fig 1. Cumulation curve of expected (grey scatter line) and observed (dark grey scatter

365 line) species of fungi on the egg shell (ES) of *Caiman latirostris* eggs.

366 Fig 2. Cumulation curve of expected (grey scatter line) and observed (dark grey scatter

367 line) species of fungi on the membrane (ESM) of *Caiman latirostris* eggs.