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Research article

# Presence of *Leptospira* spp. in *Caiman latirostris* (Crocodylia, Alligatoridae) populations in Santa Fe, Argentina

Jazmín Bauso [a,b,c], Melina S. Simoncini [a,c], Yosena Chiani [d], María F. Schmeling [d], Alejandro Larriera [c,e], Norma B. Vanasco [b,d], Carlos I. Piña [a,c,*]

[a] CICYTTP (CONICET- Prov. de Entre Ríos-UAdER), FCyT, España 149, CP 3105, Diamante, Entre Ríos, Argentina
[b] Facultad de Bioquímica y Ciencias Biológicas, UNL, Ciudad Universitaria, Paraje El Pozo, CP 3000, Santa Fe, Argentina
[c] Proyecto Yacaré-Laboratorio de Zoología Aplicada: Anexo Vertebrados (FHUC-UNL/MASPyMA), Av. Aristobulo de Valle 8700, CP 3000, Santa Fe, Argentina
[d] Instituto Nacional de Enfermedades Respiratorias (INER) "Dr. Emilio Coni", Av. Blas Parera 8260, CP 3000, Santa Fe, Argentina
[e] Facultad de Humanidades y Ciencias, UNL, Ciudad Universitaria, Paraje El Pozo, CP 3000, Santa Fe, Argentina

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ABSTRACT

Leptospirosis is a disease caused by pathogenic spirochetes of the genus *Leptospira*, transmitted by wild and domestic animals. Rodents play a fundamental role in the transmission cycle of this zoonosis but the function of reptiles is unknown. For example, crocodilians could play an important role in the transmission of this disease by living in ideal environments (bodies of shallow water and high temperatures) for the colonization of this bacterium. However, few studies have documented the presence of zoonotic diseases in caiman populations. Our objective was to assess the prevalence of antibodies to leptospira and the presence of *Leptospira* spp. in wild and captive *Caiman latirostris*. Blood samples were taken from 45 individuals (20 wild and 25 captive). Before extraction, we cleaned each caiman's neck in order to prevent contamination of samples. We determined the presence of antibodies in serum by microscopic agglutination test (MAT) and polymerase chain reaction (PCR) to detect DNA of the bacteria. We excluded 9 of the 45 samples analyzed by MAT because 5 had lipemic serum and 4 were contaminated (colonized by other organisms). Of the 36 caimans studied by microscopic agglutination test (MAT), 56% (20/36) were considered reactive (titers $\geq$50). In 74% (14/19) of captive samples and 35% (6/17) of wild samples, antibodies to leptospira were detected by MAT. The serogroup with highest occurrence was Pyrogenes (85%, n = 17/20), presenting coagglutinations with Icterohaemorrhagiae (25%, n = 5/20). One sample from a captive animal was positive for PCR, and we could not isolate leptospires because of agar contamination. Of the 45 blood agar media, 17.8% were contaminated and the rest were negative. This work determined the presence of *Leptospira* spp. in one caiman and a high prevalence of antibodies in captive caiman relative to wild individuals.

## 1. Introduction

Leptospirosis is recognized as a globally distributed bacterial zoonosis (Adler and de la Peña Moctezuma, 2010), being more common in tropical and subtropical areas with high precipitation rates (Vanasco and Sequeira, 2000; Levett, 2001; Tsegay et al., 2016). The occurrence of epidemic cases and outbreaks are determined by numerous environmental, social and economic factors. Geography favours the availability of animal reservoirs and environmental conditions favour the survival of the bacteria. Finally, socio-economic status increases the risk of exposure of people to sources of infection, both at work and in their homes (Costa et al., 2015). This zoonosis is caused by pathogenic spirochetes belonging to the order Spirochaetales, family Leptospiraceae, genus *Leptospira* (Torres-Castro et al., 2018).

*Leptospira* require temperature conditions between 25 and 32 °C, and a neutral or slightly alkaline pH (Levett, 2001). The infectious agent is transmitted from one carrier animal to another by direct or indirect contact with urine or other body fluids containing viable leptospires (Bharti et al., 2003; McBride et al., 2005; Yupiana et al., 2019). Leptospires are maintained in nature by chronic renal infection of

* Corresponding author.
*E-mail address:* cidcarlos@infoaire.com.ar (C.I. Piña).

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asymptomatic carrier animals, which eliminate the microorganism in the urine (leptospiruria) contaminating the environment. In urine, leptospires are present 4–10 days after the onset of clinical signs and the duration of excretion is variable (Levett, 2001, 2004). These bacteria do not survive in acidic urine but remain viable in alkaline urine, which is why the animals whose diets produce this type of urine are important disseminators (Adler and de la Peña Moctezuma, 2010).

Several animals (wild and domestic), as well as accidentally humans, are involved in the leptospirosis infection cycle (Torres-Castro et al., 2018). The role of reptiles in the transmission of pathogenic leptospires is unknown (Faine et al., 1999), however antibodies to leptospira have been found in several reptile species (Rossetti et al., 2003; Oliveira et al., 2016; Rodrigues et al., 2016; Pérez-Flores et al., 2017; Paz et al., 2019). *Caiman latirostris* inhabits large wetlands, which are home of a rich diversity of fauna (Larriera and Imhof, 2006), and which provide appropriate conditions for the transmission of this disease. *Caiman latirostris* is managed by a sustainable management program, where local people are involved with nest identification and egg collection, and researchers of Proyecto Yacare are in charge of incubation and assistance at hatching, so caiman could be a source of infection to humans in the program. In this work we evaluate the presence of pathogenic leptospires in wild and captive *C. latirostris* in Santa Fe Province, Argentina. In addition we also determined the pH of the urine of captive animals to determine if they could disseminate this spirochete.

## 2. Materials and methods

This research has the approval of the ethics committee of the Universidad Nacional del Litoral - Facultad de Bioquímica y Ciencias Biológicas, for animal use (Resolution 15/16). Samples were collected from caimans captured in the wild and others raised in captivity in Proyecto Yacare breeding pools at EZE-Granja La Esmeralda, Santa Fe city (31° 35′ 13.34″S, 60° 41′ 29.69″W). Sampling in the wild was carried out in two areas: El Fisco Managed Natural Reserve (30° 11′ 53.74″S, 61° 0′ 44.26″W, San Cristobal Department); and, El Estero Multiple Uses Reserve (30° 2′ 48″S, 59° 58′ 24″W, San Javier Department) in Santa Fe Province (Figure 1). These sites are within the Proyecto Yacare management program working area.

Blood samples were obtained during December, January and February (the most active months for these animals), from 2014 to 2017, from 25 captive and 20 wild individuals. At capture, wild and captive animals were examined for external signs of disease, sex determined by cloacal inspection (Brazaitis, 1969), and biometric data recorded [total length (TL) and snout-vent length (SVL) with metric tape and weight (0.01 kg precision) using a scale]. Blood was extracted from the occipital supra-vertebral sinus using 10 ml syringes (Myburgh et al., 2014), then deposited in two vacutainers (BD Vacutainer®), one containing heparin, and used for culturing, and the other containing a serum separator and used in polymerase chain reaction (PCR) and microagglutination (MAT) techniques. All analyses were done in the "Dr. Emilio Coni" laboratory at the National Institute of Respiratory Diseases (INER/CONI). Urine was obtained from captive caimans in a sterile container, and pH was measured with Biopack® (Sistemas Analíticos SA, Argentina) brand pH tapes in the range of 0–14, in order to evaluate whether these animals could become disseminators of these spirochetes.

### 2.1. Culture

Semi-solid culture media EMJH (Ellinghausen and McCullough, modified by Johnson and Harris, 1967) and Fletcher were used for the isolation of *Leptospira* spp. For the development of the technique, two cultures of blood were introduced in each tube and incubated at 28 °C for 4 months. Leptospire growth is relatively slow, with a cell doubling time of 6–8 h. Cultures were observed under darkfield microscope weekly during the first month and monthly up to 4 months.

### 2.2. Real-time PCR

Genomic DNA extraction was performed from 200 μl of serum samples, using the commercial QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's recommendation. The amplification was directed to the detection of the LipL32 gene (present only in pathogenic *Leptospira*), using the primers LipL32—45F (50 -AAG CAT TAC CGC TTG TGG TG-30) and LipL32—286R (50 -GAA CTC CCA TTT CAG CGA TT-30), which generates a fragment of 242 bp, which was detected by the probe, LipL32-189P (FAM-5′-AA AGC CAG GAC AAG CGC CG-3′ BHQ1) (Stoddard et al., 2009). We used 25 μl of final solution containing 200 nM of each primer, and 5 μl template DNA. The amplification protocol consisted of 8 min at 95 °C, followed by 45 cycles of amplification (95 °C for 3 s and 58 °C for 15 s), finishing with a cool cycle of 45 °C for 90 s (Stoddard et al., 2009). Any exponential curve with a cycle threshold (Ct) less than 40 was considered positive.

### 2.3. Microscopic agglutination test (MAT)

Sera were obtained by centrifugation at 2,700 G for 10 min, aliquoted into Eppendorf® tubes and stored at -20 °C until serology was performed. Antibodies to leptospira were detected by MAT as recommended by the World Organization for Animal Health (OIE, 2018), with some modifications, and the serum endpoint dilution was at 1:50. The MAT panel consisted of 15 serogroups maintained in the collection of the INER/CONI laboratory; Sejroe, Pyrogenes, Panama, Hebdomadis, Australis, Bataviae, Ballum, Autumnalis, Icterohaemorrhagiae, Canicola, Tarassovi, Grippotyphosa, Javanica, Cynopteri and Pomona. Each serum sample was initially diluted at 1:25 in saline, and a 50 μL aliquot of this dilution was added to 96-well flat-bottomed vinyl microplates (Costar, Corning, NY, USA). Continuously, the same volume of each corresponding antigen was added to the wells, with a final dilution of 1:50. Buffered saline solution (0.9% NaCl) was used as negative control for each reaction. Plates were incubated at 37 °C for 1h. Readings were performed under optical microscopy with a dark-field. When a sample reacted with more than one serovar, the serovar providing highest antibody titer could be the infecting serovar.

### 2.4. Statistical analysis

In order to compare the proportion of individuals with antibodies to leptospira between the two conditions (captivity and wild) we used a Bayesian approach, with normal and noninformative prior distribution (Beta distribution for presence of leptospira according to the condition), to calculate the 95% Bayesian Credible Intervals (95% BCIs) with the mean and the 2.5th and 97.5th percentiles. Differences were identified when BCIs did not overlap and/or p value was $<0.05$. All analyses were implemented in the R 3.3.3 program (R core team 2017).

## 3. Results

### 3.1. Cultures

Of the 45 blood samples sown in the two culture media, 8 samples were contaminated (6 wild and 2 captive) and the remaining 37 were negative (14 wild and 23 captive).

### 3.2. Real time PCR

We detected LipL32 gene in one sample of the 25 DNA samples of the captivity group (Ct = 35.97; indicating 2.1 leptospires/ml). None of the wild animals was positive.

### 3.3. Microscopic agglutination test (MAT)

Only 36 were analyzed by MAT (17 wild and 19 captive, Table 1) because 4 samples were contaminated and 5 presented turbidity, making observation under a dark field microscope impossible. Of the 36 samples analyzed, 20 were positive 35%, (6/17) of wild and 74% (14/19) of captive. Wild (35%) and captive (74%) animals presented significantly different proportions of individuals with antibodies to leptospira (ZBayes = 2.39; p = 0.0084). The serovars detected were: Pyrogenes, Icterohaemorrhagiae and Canicola. All captive animals presented antibodies to Pyrogenes, and four of them were also positive for Icterohaemorrhagiae (RGA). Icterohaemorrhagiae was only detected in animals from a single pool, while Pyrogenes was detected in animals from all pools. Wild animals presented antibodies to Canicola, Pyrogenes and to a lesser degree Icterohaemorrhagiae.

### 3.4. Urine pH

The pH of urine was between 7 and 8 (mean = 7.6; SD = 0.5).

## 4. Discussion

The present study records for the first time *Leptospira* spp. in both wild and captive caiman in Santa Fe Province. Research on infectious diseases in wild reptile populations is scarce (Fernández et al., 2018), and most published reports on infectious diseases correspond to animals kept in captivity (Jacobson, 1993a, 1993b). The most reported zoonotic disease in reptiles is salmonellosis (Mermin et al., 2004; Ebani, 2017), but diseases such as leptospirosis have been underestimated as a disease that could be transmitted by reptiles (Faine et al., 1999). However, the lack of sampling and the difficulty to detect mortalities in the wild may reflect a false low incidence of pathologies in these populations (Jacobson, 1993a, 1993b). More specifically, there are only four published studies on leptospires in crocodilians: Rossetti et al. (2003) with wild and captive *Caiman latirostris* and *Caiman yacare* in Chaco Province (Argentina); Pereira de Olivera (2014) in Brazil with wild *Caiman crocodilus*; Pérez-Flores et al. (2017) in Mexico with wild *Crocodylus acutus* and *Crocodylus moreletii*; and, Paz et al. (2019) in Brazil with captive *Caiman latirostris*.

Negative results in cultures could be due to the difficulty to isolate leptospires, the low sensitivity of the method (false negatives) or the absence of bacteria in the blood of the studied caimans (true negatives; Levett, 2001; Bharti et al., 2003). With respect to the real-time PCR technique, the sample of a captive individual was positive. This confirms the diagnosis in the early phase of the disease, when the bacterium is present in the blood of the animal. The culture of this sample was contaminated, so it was not possible to isolate leptospires. In addition to this, the MAT was negative, indicating that this animal should have a recent infection, and antibodies would not have increased at the time of extraction. Unfortunately, there was no second sample to observe for the presence of antibodies, because both captive and wild animals were not recaptured. We emphasize the absence of data on the leptospiremic phase in these animals, and the importance of experimental research aimed at elucidating the period of circulation of the organism in their blood (Ebani, 2017).

The MAT has the advantage of being specific. Antibodies to other bacteria do not usually cross-react with leptospira to any significant extent. However, there is significant serological cross-reactivity between leptospira serovars and serogroups (Levett, 2003; Goris et al., 2012). Thus, an animal infected with a serovar is likely to have antibodies against the infecting serovar that cross react with other serovars (usually at a lower level). Although their specificity is high, their sensitivity declines as time passes between infection and sample collection (Cumberland et al., 1999). However, for a more accurate diagnosis, two samples should be available to compare, and making possible a correct

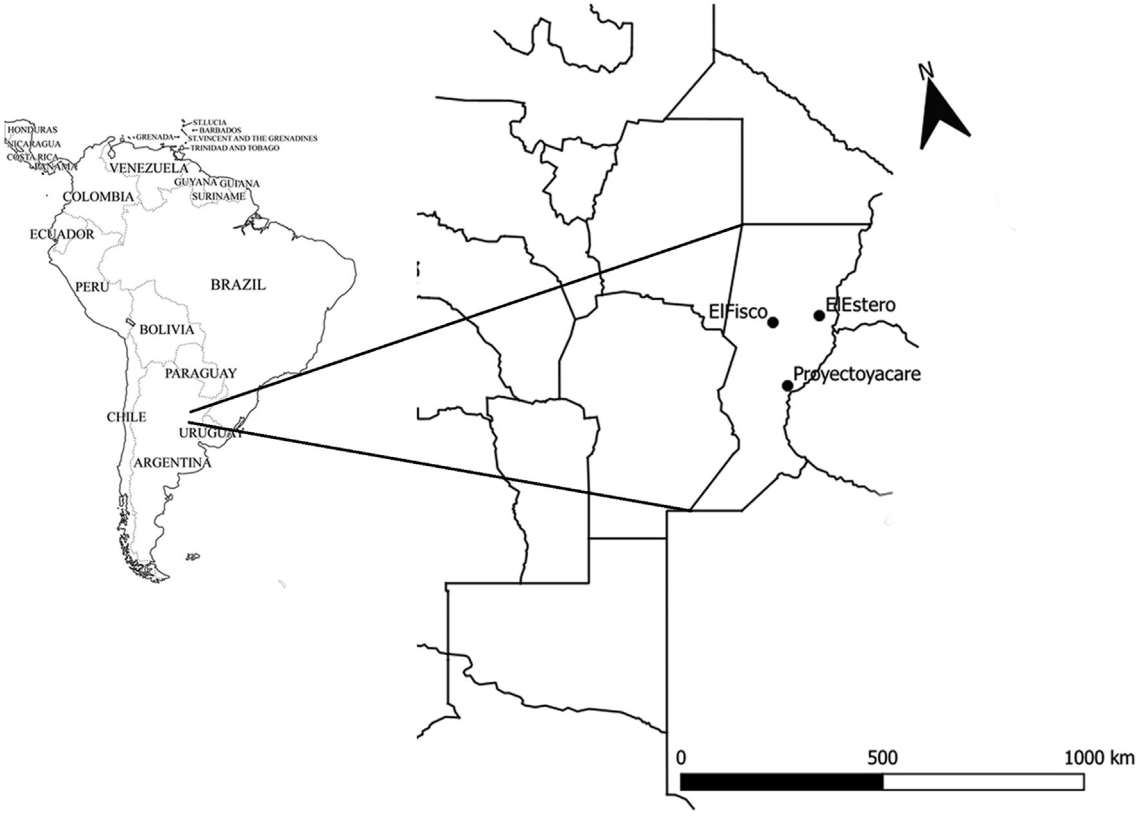


**Figure 1.** Location of study areas of *Caiman latirostris*'s in the province of Santa Fe, Argentina. Reserva Natural Manejada El Fisco; Reserva de Usos Múltiples El Estero; Proyecto Yacare.

**Table 1.** Number of *Caiman latirostris* (captive and wild animals) testing positive for *Leptospira* using MAT according to serogroup/strain and titers.

| Leptospira serogroup/strain | Captive animals n (titer) | Wild animals n (titer) |
|---|---|---|
| Canicola/Hond Utrecht IV | | 4 (1:50) |
| Icterohaemorrhagiae/RGA | 3 (1:50) 1 (1:100) | 1 (1:100) |
| Pyrogenes/Salinem | 5 (1:100) 3 (1:200) 3 (1:400) 2 (1:800) 1 (1:1600) | 3 (1:200) |

interpretation about titers variations over time. It is to emphasize that because of the type of animals sampled this is almost impossible.

Antibodies detected by MAT in captive animals belonged to the Pyrogenes serogroup and to a lesser extent to the Icterohaemorrhagiae (RGA) serogroup. Each serogroup has its preferred maintenance host to which it adapts. Many serogroups are associated with certain animal species, for example the presence of positive serology to the serogroup Icterohaemorrhagiae would indicate a high degree of cohabitation between caimans and rats (*Rattus rattus*), since this species is the natural carrier of this serogroup (Rossetti et al., 2001; Yupiana et al., 2019). In addition, the high binder titers found in some animals would suggest recent infection processes, but paired samples should be obtained to determine whether there is an increase or decrease in titers. Due to these results, Pyrogenes could be considered to be the presumably infectious serogroup because it is present with the highest titers (1:50 to 1:1600).

With respect to wild animals, low antibody titers to different strains were detected, which according to Acha and Szyfres (2001) would indicate residual antibodies from a past infection or a recent infection where antibodies in formation would be increasing. In this case, although there was no symptomatology compatible with leptospirosis at the time of sampling, it was not possible to perform serial MAT tests or specific tests to determine at what stage of infection the seropositive animals were. These results demonstrate that at the time of the sample extraction, the animals have had contact with the spirochete showing that the bacterium is distributed both in captivity and in the wild.

The presence of positive serology to the serogroups Canicola, Icterohaemorrhagiae and Pyrogenes in wild animals reflect the diversity of vectors to which animals may be exposed in the wild. Unlike the serogroups Icterohaemorrhagiae and Pyrogenes, that were present in captive animals, Canicola was only detected in wild animals. The most important hosts of the serogroup Canicola are canines (Jimenez-Coello et al., 2008; Ebani, 2017), indicating that wild animals would be in touch with canids carrying this serogroup. Foxes are known to be predators of caiman eggs (Campos and Mourão, 2015) and they have an indirect contact with female caimans.

The serogroup with the highest titer was Pyrogenes, and this same serogroup was registered by Pereira de Olivera (2014) in Brazil, Rossetti et al. (2003) in Chaco Province, and Pérez-Flores et al. (2017) in Mexico. This would confirm a wide distribution of this serogroup, found in tropical and subtropical climates. In addition to this, a study in Argentina has reported the presence of Pyrogens in humans (Chiani et al., 2016), this is another evidence of the existence of this serogroup in our country. Thus, it could be assumed that there would be a possible maintenance host capable of infecting both humans and caimans.

We observed that the percentage of wild animals that presented antibodies to leptospira (35%) was lower in relation to those in captivity (74%). This was expected due to high densities and temperatures involved with intensive rearing facilities. If we make a comparison with the work done by Pérez-Flores et al. (2017), in which they use the same cut-off titer (1:50), the seroprevalence observed in our wild animals is lower than the results they reported. In the case of the work carried out by Paz et al. (2019), the titer considered was $\geq$1:100 obtaining 95.6% of animals in captivity with antibodies to leptospira, which would also mean that the seroprevalence found in our study (74%) is lower.

Caiman urine pH was between 7 and 8 (neutral to slightly alkaline pH), and would thus be a potential disseminator of the bacteria. According to what was observed in captivity where all the animals were infected with the same serovar (Pyrogenes), two situations could be assumed: 1) that there is a contagion between individuals; or, 2) by cohabitation with rodents. These two situation are possibly because caimans cohabitated in the same pool (enclosure), therefore, share the same water source and food. We emphasize the absence of data regarding the leptospiremic phase in these animals and how their immune response would be, because from what we could observe in our study, animals with highest titers showed no symptoms of infection.

Although there are many authors who studied the presence of antibodies to leptospira in reptiles, MAT is not yet validated in these animals. However, the World Organization for Animal Health (OIE, 2018) has designated the MAT as the reference technique for the serological diagnosis of leptospirosis, and is accepted for infection prevalence and surveillance studies in animals where it has not been validated. If we compare with other studies conducted in reptiles (Rossetti et al., 2003; Grimm et al., 2015; Rockwell et al., 2019) we see that the cut-off titer used was 1:25, in our study we decided to be more conservative by establishing the dilution of 1:50 as the cut-off point since lower values can produce results of low specificity, increasing false positives (Oliveira et al., 2016). As in this study samples were obtained at a specific time, it is not possible to infer about the course of the disease, since very little is known about the pathogenesis of leptospirosis in reptiles (Ebani, 2017).

The percentage of positive reactions to the microscopic agglutination and the appearance of these serovars in wild and captive caimans highlight their importance as possible sources of infection for humans. Previous studies have reported cases of leptospirosis in people involved in alligator management activities (Feuer and Domash-Martinez, 2011), however after almost three decades of work by Proyecto Yacare operators and researchers, who carry out their activities with these animals, there have not been any reported cases of leptospirosis. According to Boadella et al. (2011), monitoring is necessary to identify changes in disease prevalence and to measure the impact of possible interventions. In addition, wildlife health monitoring generates information that benefits at least three sectors; animal health, public health and conservation. It also provides information to assist in the development and restructuring of health surveillance plans by this and other institutions dealing with these animals, and for the conduct of future related research.

## 5. Conclusion

The proportion of positive animals by the MAT technique was lower in the wild than in captivity, it is expected that under intensive breeding conditions the diseases would have a higher incidence compared to wild populations. On the basis of our results, we consider it important to continue with research aimed at elucidating the period of circulation of the bacteria in the blood of these animals and show if it possible that the reptiles were potential maintenance hosts and disseminators of the bacteria.

## Declarations

### *Author contribution statement*

Jazmín Bauso: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Melina S. Simoncini, Carlos I. Piña: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yosena Chiani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

María F. Schmeling, Norma B. Vanasco: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Alejandro Larriera: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Competing interest statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

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