



## Correspondence

### DNA-validated parthenogenesis: first case in a captive Cuban boa (*Chilabothrus angulifer*)

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Parthenogenesis is a natural form of asexual reproduction in which offspring are produced from unfertilised eggs (NEAVES & BAUMANN 2011, VAN DER KOOI & SCHWANDER 2015). This uncommon reproductive strategy has been reported in less than 0.1% of vertebrate species, nevertheless including a wide range of taxa (i.e. fishes, amphibians, reptiles, birds and mammals), even in wild populations (NEAVES & BAUMANN 2011, BOOTH et al. 2012, VAN DER KOOI & SCHWANDER 2015, DUDGEON et al. 2017, ALLEN et al. 2018). Parthenogenetic reproductive events have caught the attention of evolutionary and conservation biologists since the absence of genetic recombination accelerates the accumulation of deleterious mutations in parthenogenetic individuals, which may also have implications in the management and conservation of the species (ENNOS et al. 2005, HEDRICK 2007, LAMPERT 2008). In fact, parthenogenesis has been related to an increased incidence of abortive events, stillbirths, and embryonic and developmental abnormalities (e.g. anophthalmia, microphthalmia, encephalocoele and head foreshortening) in several species (BILLY 1986, BOOTH & SCHUETT 2016, ALLEN et al. 2018).

Obligate parthenogenesis is a biological process in which all individuals within a species reproduce asexually (VAN DER KOOI & SCHWANDER 2015, BOOTH & SCHUETT 2016). Among vertebrates, this reproductive strategy is restricted to Squamate reptiles, being reported in various lizard species and one snake (*Indotyphlops braminus*) (WYNN et al. 1991, KEARNEY et al. 2009, BOOTH & SCHUETT 2016). The occasional occurrence of parthenogenesis in individuals of a species that normally reproduces sexually (i.e. facultative parthenogenesis – FP) was first mentioned in birds in the late 1800s (OELLACHER 1872). The phenomenon of

FP has been reported in various species of major vertebrate groups including reptiles, birds and elasmobranchs (sharks and rays) (BOOTH & SCHUETT 2016, HARMON et al. 2016, DUDGEON et al. 2017, RAMACHANDRAN & MCDANIEL, 2018). Most FP events were documented in captive females after long periods without contact with male conspecifics during their reproductive lifetime (LAMPERT 2008, BOOTH & SCHUETT 2016). However, parthenogenesis has recently been reported in both wild snake populations (BOOTH et al. 2012, CALVETE et al. 2018) and females housed with males (BOOTH et al. 2014), suggesting that its occurrence may be more frequent in vertebrates than previously thought. In addition, the reproductive viability of parthenogenetic offspring has been observed in some species, which highlights the ecological and evolutionary significance of this reproductive strategy (CALVETE et al. 2018). Nevertheless, the biological basis and mechanisms underlying parthenogenesis remain mostly unknown (BOOTH & SCHUETT 2016, DUDGEON et al. 2017, SHIBATA et al. 2017).

Accurate identification and characterization of parthenogenesis in captive individuals of non-model species may provide important data towards understanding the frequency, causes, consequences and biological mechanisms of asexual reproduction among vertebrates (FUJITA & MORITZ 2009, NEAVES & BAUMANN 2011). The occurrence of FP has been described in at least six snake families, namely Boidae, Pythonidae, Viperidae, Acrochordidae, Colubridae and Elapidae (BOOTH & SCHUETT 2016, SHIBATA et al. 2017, ALLEN et al. 2018). Among boid snakes (Boidae family), parthenogenesis has been recorded and validated by genetic analysis in *Boa constrictor* and *Eunectes murinus*, as well as in two species of the genus *Epicrates* (*E. maurus*

and *E. cenchria*), closely related to the genus *Chilabothrus* (BOOTH et al. 2011a, 2011b, KINNEY et al. 2013, SHIBATA et al. 2017). Various FP events confirmed by genetic analysis have also been reported in Pythonidae (*Python bivittatus*, *P. regius* and *Malayopython reticulatus*, GROOT et al. 2003, BOOTH et al. 2014), Viperidae (*Agkistrodon contortrix*, JORDAN et al. 2015, CALVETE et al. 2018), Colubridae (*Thamnophis marcianus* and *T. couchii*, GERMANO & SMITH 2010, REYNOLDS et al. 2012), and Elapidae (*Oxyuranus scutellatus* and *Acanthophis antarcticus*, ALLEN et al. 2018). The application of molecular techniques greatly improved the characterization of FP events in wild and captive snakes. However, to understand the evolutionary significance of this alternative reproductive strategy it is essential to increase the effort to detect evidence of new potential FP occurrences.

Here, we report two interesting reproductive events recorded in a captive female Cuban boa (*Chilabothrus angulifer*) isolated from males for 11 years. These occurrences could be explained by two hypotheses: (i) long-term sperm storage from the last mating event or (ii) parthenogenetic reproduction. We applied molecular and histopathological methodologies to evaluate both hypotheses, providing the first evidence of facultative parthenogenesis in a Cuban boa.

On September 20, 2017, a 20-year-old captive female Cuban boa from the Zoo da Maia (Maia, Portugal) collection gave birth to a stillborn and deposited multiple non-embryonated eggs. This female, added to the zoological collection in 1999, has had no contact with a male since 2006 when its mate died. Previously, in 2013, this female had delivered a yellowish mass of non-developing eggs and a dead neonate, that has been preserved in 10% buffered formalin. The offspring from these two deliveries, one fresh and the other formalin-fixed, were analysed in the Histology and Anatomical Pathology Laboratory of Trás-os-Montes e Alto Douro University (UTAD). Tissues samples were processed for histopathology applying the routine staining techniques, with haematoxylin and eosin (HE), for light microscopy.

The DNA isolation from the formalin-fixed specimen (neonate 2013) was carried out using the Quick-DNA Miniprep Plus Kit (Zymo Research), according to the manufacturer's recommended protocol, with some additional steps before sample digestion. Briefly, a mixture of different tissues (liver, lung, gut and skin) was sliced into small pieces with a scalpel. Then the tissues were washed with Phosphate-buffered saline (PBS) during 24 h (the buffer was replaced twice). The DNA extraction from muscle tissues of the neonate born in 2017 was performed using the NZY Tissue gDNA Isolation kit (Nzytech). The mother's DNA was isolated from her blood using the NZY Blood gDNA Isolation kit (Nzytech). Both extractions were performed following the standard protocols recommended by the manufacturer.

Thirteen microsatellite markers previously characterized for boid species were analysed:  $\mu$ sat 1,  $\mu$ sat 10,  $\mu$ sat 13,  $\mu$ sat 24,  $\mu$ sat 32,  $\mu$ sat 36, Ci25, Ci34, Ci35, Ci36, Ci37, 55HDZ554, and 55HDZ617 (RAMANANA et al. 2009, TZIKA et al. 2009, BOOTH et al. 2011b, REYNOLDS et al. 2014). Pre-screening of microsatellite variations between mother and

offspring samples was performed using high-resolution melting (HRM) analysis (MADER et al. 2008, LOMBAL et al. 2015). PCR amplification and melting acquisition were carried out using a QuantStudio 3 Real-Time PCR System (Applied Biosystems). The reaction mixture was prepared to a 20  $\mu$ l final volume containing 10  $\mu$ l of MeltDoctor HRM Master Mix (Applied Biosystems), 5 pmol of each primer and 5 ng of genomic DNA. All PCR reactions were performed in duplicate. The amplification protocol was run as follows: 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min (fluorescence signal was captured at the end of each cycle); 1 cycle of 95°C for 15 s, 60°C for 1 min and then sequential temperature increments of 0.025°C/s with temperature ranging from 60°C to 95°C, with continuous fluorescence measurements. The melting curve data were analysed using the QuantStudio Design & Analysis software v.1.4 (Applied Biosystems) and the High Resolution Melting (HRM) Software v.3.0.1 (Thermo Fisher Scientific). The differences in melting-curve shapes allowed the characterisation of the microsatellite allelic variability. Forward primers of the microsatellites with variations among samples were labelled with 6-FAM to determine the genotypes using capillary electrophoresis. PCR amplifications were performed to a total volume of 20  $\mu$ l containing 10  $\mu$ l of 2  $\times$  MyTaq HS Mix (Bioline), 5 pmol of each primer and 5 ng of DNA. PCR thermal conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 30 s and a final extension at 60°C for 10 min. Amplified fragments were submitted to electrophoresis on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) using the GeneScan 500 LIZ size standard. Allele sizes were determined using Peak Scanner v.3.0.2 (Thermo Fisher Cloud).

Our results support the first evidence of FP in the Cuban boa (*Chilabothrus angulifer*). Macroscopically, both reptiles corresponded to fully developed stillborn snakes that showed no morphological alterations except in the 2017 specimen (#2), which presented bilateral anophthalmia (Fig. 1). Microscopic examination of organs showed no alterations and the inspection of the reproductive system confirmed both to be female. Of the 13 microsatellite loci screened, two markers ( $\mu$ sat 13 and Ci34) did not amplify or they generated non-specific PCR products. The high-resolution melting (HRM) analysis allowed the identification of four microsatellite loci ( $\mu$ sat 10,  $\mu$ sat 24, Ci36 and Ci37) with allelic variability between mother and offspring samples. No evidence of allelic variability was detected in the remaining loci for the samples analysed. These results were validated using capillary electrophoresis to determine allele sizes for all polymorphic markers and for two non-polymorphic loci (Table 1). Maternal heterozygosity was observed for polymorphic loci and a homozygosity pattern was obtained for the non-polymorphic loci analysed (Table 1). The offspring were homozygous for all microsatellite loci, always carrying an allele present in the mother (Table 1). The locus Ci37 is a potential null allele in the neonate of 2013 since the amplification failed using different DNA samples and PCR conditions.

Table 1. Genotypes of mother and offspring characterised for the potentially parthenogenic Cuban boa (*Chilabothrus angulifer*)

Individual	$\mu$ sat 10	$\mu$ sat 24	Ci35	Ci36	Ci37	55HDZ617
Mother	401/409	215/217	318/318	220/228	278/298	185/185
Neonate 2013	409/409	217/217	318/318	220/220	–	185/185
Neonate 2017	409/409	215/215	318/318	228/228	298/298	185/185

The allele homozygosity in the offspring, carrying only maternal alleles, was similar to previous FP cases reported for boid species (BOOTH et al. 2011a, 2011b, KINNEY et al. 2013, SHIBATA et al. 2017). In the Boidae family (for genera *Boa* and *Epicrates*), parthenogenesis was initially detected in all-female litters (BOOTH et al. 2011a, 2011b). Similarly, the two stillborn analysed in this study were both females. Accidental FP in captive individuals generally occurs after long periods of isolation from mates (BOOTH & SCHUETT 2016). The female in this study has had no contact with any conspecific male for 13 years now. Prolonged sperm storage has been documented in various snake species, but the longest time period of suspected sperm storage reported for a snake was seven years and six months (MAGNUSSON 1979, BIRKHEAD & MØLLER 1993, BOOTH & SCHUETT 2011). In our case we described the lack of male genetic contribution to the offspring using molecular analysis, thus excluding prolonged sperm storage. The high levels of homozygosity detected in the offspring are a characteristic of the parthenogenetic mode explained by terminal fusion automixis (BOOTH et al. 2011a, 2011b), as recently inferred for long-term captive copperheads (*Agkistrodon contortrix*) and cottonmouths (*A. piscivorus*) (SHIBATA et al. 2017, ALLEN et al. 2018).

Genome-wide homozygosity of parthenogenetic offspring may be related to the development of malformations (BOOTH et al. 2012). Embryos and stillborn offspring with developmental abnormalities have been associated with parthenogenetic events in reptiles (BILLY 1986, BOOTH & SCHUETT 2016, ALLEN et al. 2018). The morphological evaluation of the Cuban boa neonate born in 2017 evidenced bilateral anophthalmia, a malformation found in parthenogenetic offspring of other reptile species (BILLY 1986).

In conclusion, we identified and described a probable first record of FP in the Cuban boa supported by specimen history and molecular markers. This may have important ecological and evolutionary implications, including the improved understanding of this reproductive strategy and its frequency in captivity, and possibly in the wild, as recorded for some species (BOOTH et al. 2012, FIELDS et al. 2015, CALVETE et al. 2018). The increasing number of reports on parthenogenic births in a wide range of snakes and other vertebrates also points to the evolutionary significance of this reproductive phenomenon, as yet poorly understood, making it a research field with high potential (LAMPERT 2008, AVISE 2015). Therefore, we encourage zoo workers, veterinarians, curators, wildlife managers and researchers to pay attention and to investigate further evidence towards abnormal births in this species and related taxa, since these events may be easily overlooked.

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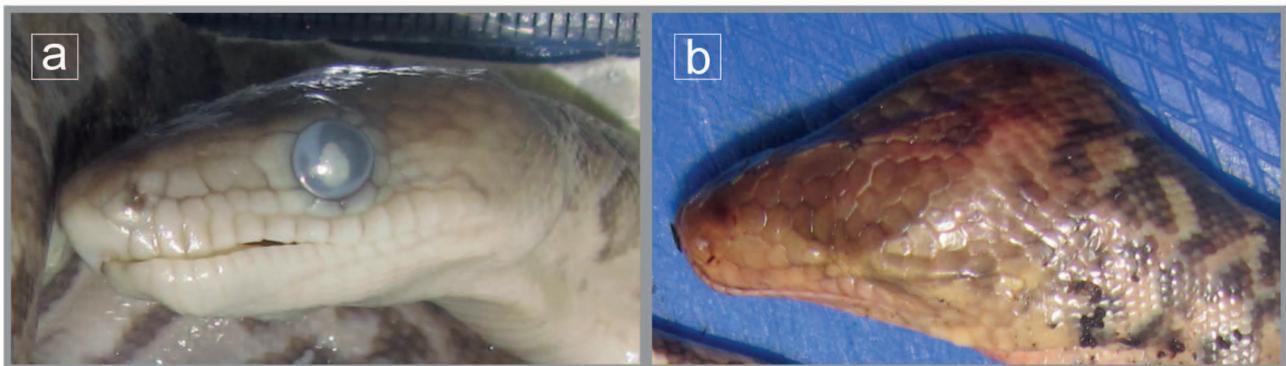


Figure 1. Neonates of the Cuban boa (*Chilabothrus angulifer*): (a) Neonate from 2013 with a normal head; (b) Neonate from 2017 with bilateral anophthalmia.

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