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Consecutive Virgin Births in the New World Boid Snake, the Colombian Rainbow Boa, *Epicrates maurus*

Warren Booth, Larry Million, R. Graham Reynolds, Gordon M. Burghardt, Edward L. Vargo, Coby Schal, Athanasia C. Tzika, and Gordon W. Schuett

From the Department of Entomology and W. M. Center for Behavioral Biology, North Carolina State University, Raleigh, NC 27695-7613 (Booth, Vargo, and Schal); Fort Lauderdale, FL (Million); the Department of Ecology and Evolutionary Biology, The University of Tennessee, Knoxville, TN (Reynolds and Burghardt); the Department of Genetics and Evolution, University of Geneva, Sciences III, Genève 4, Switzerland (Tzika); and the Department of Biology and Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA (Schuett).

Address correspondence to W. Booth at the address above, or e-mail: wbooth@ncsu.edu.

Until recently, facultative automictic parthenogenesis within the squamate reptiles exhibiting ZZ:ZW genetic sex determination has resulted in single reproductive events producing male (ZZ) or female (ZW) offspring. With the recent discovery of viable parthenogenetically produced female (WW) Boa constrictors, the existence of further parthenogenetic events resulting in WW females was questioned. Here, we provide genetic evidence for consecutive virgin births by a female Colombian rainbow boa (*Epicrates maurus*), resulting in the production of WW females likely through terminal fusion automixis. Samples were screened at 22 microsatellite loci with 12 amplifying unambiguous products. Of these, maternal heterozygosity was observed in 4, with the offspring differentially homozygous at each locus. This study documents the first record of parthenogenesis within the genus *Epicrates*, a second within the serpent lineage Boidae, and the third genetically confirmed case of consecutive virgin births of viable offspring within any vertebrate lineage. Unlike the recent record in Boa constrictors, the female described here was isolated from conspecifics from birth, demonstrating that males are not required to stimulate parthenogenetic reproduction in this species and possibly other Boas.

Key words: *asexual reproduction, Boidae, facultative parthenogenesis, microsatellite DNA fingerprinting, Serpentes*

Facultative parthenogenesis, the alternation between sexual and asexual reproduction, is considered extremely rare, having been reported in less than 0.1% of vertebrate species (Olsen and Marsden 1954; Olsen 1975; Chapman et al. 2007, 2008; Schut et al. 2008; Parker and McDaniel 2009; Feldheim et al. 2010). Although almost exclusively sexual, rare instances of facultative parthenogenesis have been described in the squamate reptiles (lizards and snakes) that exhibit genetic sex determination with ZW (female) and ZZ (male) sex chromosomes (Dubach et al. 1997; Schuett et al. 1997; Groot et al. 2003; Lenk et al. 2005; Watts et al. 2006; Booth et al. 2011). Facultative parthenogenesis has yet to be identified in natural populations, but there is increasing interest in understanding both the diversity of lineages within which it occurs and its frequency (Lampert 2008). Furthermore, its evolutionary significance is also of interest (Lampert 2008; Neaves and Baumann 2011) as are its implications for the conservation of endangered species (Watts et al. 2006; Chapman et al. 2007), given that populations that exhibit frequent and/or habitual parthenogenetic reproductive events may accumulate deleterious mutations through the lack of genetic recombination (e.g., Muller's ratchet) at a higher rate than those utilizing sexual reproduction (see Hedrick 2007). Instances of parthenogenesis in captive individuals have therefore proved extremely valuable in furthering our understanding of asexual reproduction in vertebrate lineages.

Parthenogenetic modes and the reproductive outcomes exhibited in the Pythonidae, Boidae, and Caenophidia (so called higher/advanced taxa) have proved unusually variable when examined with molecular markers. Within the caenophidians, which contains most extant snakes (Greene 1997; Wiens et al. 2008), small numbers of male (ZZ) offspring have been reported, resulting from terminal fusion automixis (the fusion of the second polar body with the egg nucleus) (Schuett et al. 1997). Given the heterogametic nature of the female sex chromosomes, these offspring essentially represent half-clones of the mother (Lampert 2008). In contrast, the single report of parthenogenesis within pythonids resulted in female (ZW) embryos that maintained the mother's heterozygosity (Groot et al. 2003).

The actual parthenogenetic mode in this case was undetermined; however, it was reputed to be either apomixis (repression of meiosis with oocyte production by mitosis), premeiotic doubling of chromosomes (genome doubling prior to meiosis), or central fusion (fusion of the first polar body to the egg nucleus). Recently, within boids, a female *Boa constrictor imperator* housed with up to 4 conspecific males produced 2 successive litters comprised of only female offspring with the sex chromosomal arrangement WW (Booth et al. 2011). The likely mode of parthenogenesis in this case was terminal fusion automixis.

An intriguing aspect of the parthenogenetic events in *B. constrictor imperator* described by Booth et al. (2011) was the lack of male offspring. Through terminal fusion automixis, a heterogametic female should theoretically produce equal numbers of male (ZZ) and female (WW) offspring. Although the mechanism responsible for this was undetermined, it was proposed that the adult female may have exhibited hemizygosity of the sex-determining chromosomes (i.e., W_{null}).

Although facultative parthenogenesis has been reported in a number of captive vertebrates, the production of viable parthenogenetic offspring across successive litters is rare. Indeed, only single instances in both birds and snakes document such an event. Olsen and Marsden (1954) determined that 568 eggs produced from 79 virgin female turkeys (22.4% of all eggs produced in the study) exhibited growth consistent with parthenogenetic reproduction. Through selective breeding, the production of an entire strain of parthenogenetic turkeys was later achieved (Olsen 1975), potentially suggesting a heritable component to this reproductive mode. Recently, Schut et al. (2008) identified 7 instances of parthenogenetic development in eggs produced by 3 female Zebra finches (*Taeniopygia guttata*); however, hatchling failure was recorded in all. In snakes, Booth et al. (2011) documented parthenogenetic reproduction across 2 successive litters from a female that had previously reproduced sexually.

This recent discovery of viable WW female offspring by Booth et al. (2011) upends decades of scientific theory, originally proposed by Olsen and Marsden (1954) based on research performed on domestic fowl, which suggests that the production of viable WW embryos was not possible. In subsequent studies of parthenogenesis in serpents from relatively derived lineages, this theory would appear to be supported (Schuett et al. 1997; Groot et al. 2003). Owing to the basal position of the Boidae within Serpentes (Greene 1997; Kelly et al. 2003; Wiens et al. 2008) and the homomorphic nature of the sex chromosomes (Becak 1972), understanding both the parthenogenetic mode exhibited by other genera within boid taxa, and the possible occurrence of additional examples of WW parthenogens is of particular interest (Booth et al. 2011).

Materials and Methods

Sample Description

On 3 October 2006, within a private collection (L.M.), a female Colombian rainbow boa, *Epicrates maurus*, housed in isolation after being purchased 3 days after her birth on 5 April 1987, gave birth to a litter of 10 offspring. Of these, 6 were alive, 2 deformed and later euthanized, and 2 stillborn. Deformed and euthanized samples were disposed of without tissue collection. Of the 6 that were alive, 4 subsequently died over the course of several months owing to the failure to establish feeding. These samples were preserved frozen and were available for genetic analysis. The following year, on 22 September 2007, the female produced a second litter consisting of 3 stillborn offspring. These were immediately preserved frozen. An unrecorded number of unfertilized ova were produced in each reproductive event. Three years later in 2010, the adult female died at the age of 23. Given the age at purchase, the isolation from males since purchase, and the duration of time that had passed to produce these 2 litters, long-term sperm storage was not considered a viable possibility. Furthermore, the maximum period of time for which sperm storage is considered viable in reptiles is 7 years as reported in the acrochordid (Acrochoridae) snake *Acrochordus javanicus* (Magnusson 1979; reviewed in Birkhead and Møller, 1993); however, that record is in doubt owing to the fact that parthenogenesis has been described in the congener *A. arafurae* (Dubach et al. 1997). Captive history, therefore, suggests parthenogenesis as the likely reproductive mode responsible for the production of these litters. Conclusive evidence of parthenogenesis was sought through the application of appropriate microsatellite markers to the mother, 4 offspring from 2007 and 3 from 2008. Additionally, all deceased offspring were sexed through visualization of the gonads following dissection to investigate the possible occurrence of WW females within the genus *Epicrates*.

Microsatellite Markers and Genotyping

Total genomic DNA was extracted from shed skin (mother) or muscle tissue (offspring) using the PUREGENE DNA isolation procedure (Gentra Systems Inc., Minneapolis, MN). Samples were screened at 22 loci: *E. subflavus* (*usat*-1, *usat*-20, *usat*-36) (Tzika et al. 2009; Booth et al. 2011), *usat*-2, *usat*-4, *usat*-5, *usat*-6, *usat*-14, and *usat*-32 (developed but unpublished by Tzika et al. 2009; see Table 1 for primer sequences and conditions), *Sanzinia madagascariensis madagascariensis* (*55hdz305*, *55hdz328*, *55hdz452*, *55hdz554*, *55hdz559*, *55hdz600*, *55hdz603*, and *55hdz617*) (Ramana et al. 2009), and *B. c. imperator* (*Bci*-14, *Bci*-15, *Bci*-18, *Bci*-21, and *Bci*-23) (Booth et al. 2011). Polymerase chain reactions (PCRs) followed conditions outlined by the authors with minor modifications for visualization on a Li-Cor 4300 dual laser DNA sequencer (Li-Cor Biosciences, Lincoln, NE). Amplified products were labeled with M13F-29 IRDye infrared tags (Li-Cor). Following PCR, 4 μl of stop solution (95% formamide, 20 mM EDTA, bromophenol blue) was added to each 12 μl reaction. Reactions were subsequently denatured at 90 °C for 4 min, and 1 μl was loaded onto 25 cm 6% 1X TBE polyacrylamide gels, mounted on a Li-Cor 4300 automated DNA sequencer. Loci were sized using a 50–350 bp standard (Li-Cor). Gels were run at a constant power of 40 W at 50 °C for

Table 1 Characterization of 4 microsatellite DNA loci amplifying consistent PCR products in *Epicrates maurus* (originally developed for *E. subflavus*)

Locus	Repeat motif	Sequence	T_a (°C)	$MgCl_2$	Each primer (μm.)	Fragment size (bp)
usat-2	$(CATT)^n$ + $(TATT)^n$	F: GTTTCTTCCCCAAA TTCATGCTTGACAG R: CCCCTCCTCTCCACTTCC	56	2.0	1.0	324
usat-4	$(GT)^n$	F: GTTTCTTTGA GGATTCCTTGTTTTC R: TTTTCCCCTATTTTTCCC	50	2.0	1.0	317
usat-6	$(TC)^n$	F: GTTTCTTGTTTA CCCTTCCATGCATCCTCTT R: ACGCAAACC GCCTCTCCCC	56	2.0	1.0	302
usat-32	$(ATC)^n$	F: GTTTCTTTGTTTTT CTCTTAGTCC R: TTGCTGGAGGGAGAC	50	2.5	0.6	367

Annealing temperature (T_a), Primer concentration, and PCR product size are described.

2 h. Results were analyzed using GENEPROFILER software (Scanalytics, Inc.).

Results and Discussion

Of the 22 loci screened, 12 amplified consistent products (*Bci*-14, *Bci*-15, *usat*-1, *usat*-2, *usat*-4, *usat*-6, *usat*-20, *usat*-32, *55hdz305*, *55hdz600*, *55hdz603*, and *55hdz617*). Of these, 4 proved polymorphic with maternal heterozygosity observed (Table 2). All offspring were differentially fixed for a maternal allele (Table 2). Following dissection of the deceased specimens, all offspring were found to possess ovaries and lack testes. Booth et al. (2011) reported that juvenile male *B. c. imperator* surgically examined possessed both testes and ovaries, whereas females of the same age possessed only ovaries. It is assumed that the ovaries of males degenerate prior to the onset of reproductive competence. Parthenogenetically produced females of *B. c. imperator* of a comparative age were found to possess only ovaries.

The differential homozygosity observed in the offspring at the maternally heterozygous loci, combined with the visual determination of sex as female, reflects the previous report of parthenogenesis in Boa constrictor (Boidae) by Booth et al. (2011) and confirms the occurrence of further WW females within this basal serpent lineage. The elevated homozygosity supports the likely parthenogenetic mode as being terminal fusion automixis. In contrast to the WW females described by Booth et al. (2011) and comparable to the parthenogenetic births described in other squamates (Schuett et al. 1997; Groot et al. 2003; Lenk et al. 2005, Watts et al. 2006), no males were present at any point during the life of this female. Thus, it is clear that male courtship could not have served as a stimulus for parthenogenetic reproduction in this case and therefore may not have played a role in *B. c. imperator*, as suggested by those authors (Booth et al. 2011).

Table 2 Genotypes of mother and 7 offspring at maternally heterozygous loci

Individual	*usat*-4	*usat*-6	*usat*-20	*usat*-32
Mother	317/319	302/308	323/359	367/379
2006-OS1	317/317	302/302	323/323	367/367
2006-OS2	317/317	302/302	323/323	379/379
2006-OS3	319/319	308/308	323/323	379/379
2006-OS4	317/317	308/308	359/359	379/379
2007-OS1	319/319	308/308	323/323	379/379
2007-OS2	319/319	308/308	359/359	367/367
2007-OS3	317/317	308/308	359/359	379/379

The lack of male offspring in the litters described previously by Booth et al. (2011) and those described here question the dynamics of genetic sex determination in these basal snake lineages. Relatively, little is known regarding the genes responsible for sex determination in snakes and their chromosomal locations (Ezaz et al. 2006). For example, 2 highly conserved vertebrate sexual differentiation genes, *DMRT1* and *SOX9*, located on the Z chromosome of birds, have been mapped to chromosome 2 of 3 evolutionarily diverged snake species (*Python molurus*, *Elaphe quadrivirgata*, and *Trimeresurus flavoviridis*), suggesting alternative genes are responsible for sex determination in snakes (Matsubara et al. 2006). However, *DMRT1* was mapped to the Z chromosome of the Tiger snake, *Notechis scutatus* (Ezaz et al. 2006). Considerable variation may therefore exist in the sex-determination genes within the snake families, thus cytological studies of parthenogenetically reproducing female boids and their offspring may provide a valuable insight in future research.

Of particular significance, the present results document the second recorded case of consecutive parthenogenetic births within squamata. In squamates, with the exception of Booth et al. (2011), all cases confirmed through the

application of molecular methods have documented small numbers of individuals of single reproductive events. Nonetheless, in snakes, Schuett et al. (1997) reported that a captive garter snake *Thamnophis elegans* produced 4 litters from 1988 to 1994 that were suspected of being produced parthenogenetically, but only one (1991) was confirmed by molecular methods (minisatellites). Likewise, although Groot et al. (2003) reported that the female Burmese python examined in their study laid clutches containing eggs that appeared outwardly fertile over several years, molecular methods (AFLP's) were only used to confirm those within a single clutch. A recent report of parthenogenesis in a white-spotted bamboo shark (*Chiloscyllium plagiosum*) described viable offspring produced from 7 eggs deposited over a 6-month period (Feldheim et al. 2010). Due to the reproductive biology of oviparous sharks, however, this is not considered consecutive (independent) reproductive cycles (Chapman D, personal communication).

It has been hypothesized that parthenogenesis is a reproductive error resulting from captive conditions and isolation from suitable mates (Lampert 2008). Hedrick (2007) added to this possibility by hypothesizing that the production of viable offspring through parthenogenesis would be extremely reduced in outbred individuals. Theoretically, those directly derived from wild outbred populations might be expected to possess deleterious alleles dispersed throughout their genome in a higher frequency than those from inbred and therefore potentially "purged" captive lines (Feldheim et al. 2010). At the time of purchase of the present female, captive reproduction of members of the genus *Epicrates* was in its infancy. As a result, adults and pregnant females were frequently imported for the reptile trade. The likelihood, therefore, that the adult female we describe here was purged of potentially deleterious alleles is extremely slim. Given the poor survival of the present offspring produced over 2 consecutive litters, these results support the genetic hypothesis put forward by Hedrick (2007).

In conclusion, this study adds to a handful of others successfully using molecular markers to identify facultative parthenogenesis in vertebrate species. Evidence supporting terminal fusion automixis in the genus *Epicrates* provides a second case of parthenogenesis within the lineage Boidae and represents the third study to describe consecutive parthenogenetic births producing viable offspring from a single female in any vertebrate lineage. The detection of homozygous females within a species possessing ZW:ZZ sex chromosomes supports the hypothesis of Booth et al. (2011), that WW females are indeed more common within some basal reptilian lineages, such as boids, than previously considered.

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