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Evidence for viable, non-clonal but fatherless Boa constrictors

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Parthenogenesis in vertebrates is considered an evolutionary novelty. In snakes, all of which exhibit genetic sex determination with ZZ : ZW sex chromosomes, this rare form of asexual reproduction has failed to yield viable female WW offspring. Only through complex experimental manipulations have WW females been produced, and only in fish and amphibians. Through microsatellite DNA fingerprinting, we provide the first evidence of facultative parthenogenesis in a *Boa constrictor*, identifying multiple, viable, non-experimentally induced females for the first time in any vertebrate lineage. Although the elevated homozygosity of the offspring in relation to the mother suggests that the mechanism responsible may be terminal fusion automixis, no males were produced, potentially indicating maternal sex chromosome hemizygosity (WO). These findings provide the first evidence of parthenogenesis in the family Boidae (Boas), and suggest that WW females may be more common within basal reptilian lineages than previously assumed.

Keywords: facultative parthenogenesis; asexual reproduction; Boidae; WW female; microsatellite DNA fingerprinting

1. INTRODUCTION

Sex determination in reptiles is extraordinarily diverse, and can involve temperature-dependent mechanisms or genotypic sex determination with either male or female as the heterogametic sex (i.e. XX females and XY males in at least two species of turtle and some lizards, or ZZ males and ZW females in other turtles, other lizards and all snakes [1]). In organisms with females as the heterogametic sex, the chromosomal arrangement WW, resulting in females, theoretically may occur through terminal fusion automictic parthenogenesis [2]; however, studies suggest that these zygotes fail to develop [3–6]. Only through artificially induced gynogenesis [7], a procedure involving sperm deactivation prior to fertilization, followed by the inhibition of the formation of the second polar body [8], have viable WW females been produced, and only in fish [7] and amphibians [8].

Although reproduction in snakes is almost exclusively sexual (one recorded exception being the Brahminy blind snake, *Ramphotyphlops brahminus*, a triploid, all-female species reproducing by obligate gynogenesis [9,10]), rare instances of facultative parthenogenesis in captivity have produced males (ZZ), probably through automictic terminal fusion (i.e. the fusion of the second polar body with the egg nucleus during meiosis, resulting in extensive homozygosity; [5,6,11,12]), or females (ZW) following apomixes, premeiotic doubling or automictic central fusion (i.e. fusion of the first polar body with the oocyte during meiosis, preserving maternal heterozygosity; [3,13]). Among the primitive snakes of the families Boidae (Boas) and Pythonidae (Pythons), anecdotal reports of parthenogenetic reproduction exist, but only one instance has been scientifically demonstrated [3]. Apparently fertile eggs produced by an isolated Burmese Python (*Python molurus bivittatus*) were partially incubated before embryo dissection for genetic screening and sexing. In contrast to parthenogenetic offspring produced in the more derived serpent lineages [5], these parthenogenetically produced embryos were all ZW females. The long-term viability of these females is unknown, however, as no eggs were incubated to hatching.

This single parthenogenetic event recorded in the Pythonidae resulted in embryos with levels of heterozygosity comparable to that of the mother [3]. Although genic heterozygosity is retained, essentially comparable to that of the mother, habitual parthenogenetic reproduction will eventually lead to the accumulation of deleterious mutations through the lack of recombination (e.g. Muller's ratchet). Given the close evolutionary relationship between the Boidae and the Pythonidae [14,15] and the listing of a number of species within these families as endangered under the Convention for the Trade in Endangered Species act, understanding the evolutionary breadth of parthenogenesis within the basal serpent lineages will provide valuable information for consideration if conservation strategies are developed.

In 2004, a captive-born female *Boa constrictor imperator* produced a small litter via sexual reproduction with a male *B. c. constrictor* (a closely related subspecies), while both were housed in a private collection. After the male was removed and the female was housed alone, no additional litters were produced from 2005 through 2007. In the next 2 years, the female produced two litters of live offspring (2009 = 12, 2010 = 10), coincident with being housed and possibly copulating with up to four *B. c. imperator* males. These litters were unusual because all of the offspring were female, and all exhibited a rare phenotype known as caramel, a recessive colour trait expressed by the mother, but a gene not believed to be carried in the heterozygous state by the males present. Three alterative hypothesis could explain the litters in 2009 and 2010: (i) one or more male *B. c. imperator* housed with the female since 2008 were unknowingly heterozygous for the caramel trait and contributed paternal alleles to the offspring; (ii) long-term sperm storage from the 2004 mating, with the male *B. c. constrictor* proving heterozygous for the caramel colour trait; or (iii) the litters resulted from facultative

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parthenogenesis from an otherwise sexually reproducing female. Through a combination of DNA fingerprinting and sex determination by exploratory surgery, we provide the first evidence of multiple parthenogenetically produced females in the family Boidae, and confirm for the first time that WW females within this primitive lineage are viable.

2. MATERIAL AND METHODS

(a) *Microsatellite analysis*

Genomic DNA was extracted from 1 × 3 cm sections of individual shed skins, collected from all offspring, the mother and all males with which she was housed since 2008, following the method described by Taggart *et al.* [16]. DNA was unavailable for the 2004 male and his offspring. Samples were screened and genotyped at five microsatellite loci (table 1) newly developed for *B. c. imperator* following the method outlined by Booth *et al.* [17], and at three additional loci developed by Tzika *et al.* [18].

(b) *Identification of gender*

In female *B. c. imperator*, the cloacal sacs can be probed to a depth of three to four subcaudal scales. This method has been demonstrated to accurately indicate the gender of snakes, unless there has been some alteration of normal development or damage to the area. All members of the 2009 and 2010 litters were sexed by cloacal probing. To confirm these results, a single randomly selected representative of the 2009 litter was subjected to exploratory coeliotomy to identify the nature of the gonad. For comparison, four age-matched individuals (two males and two females) from a sexually produced litter were surgically sexed using the same approach. Each snake was induced using teletamine and zolazepam (Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA), 30 mg kg^{-1} IM, intubated and maintained on sevoflurane gas (SevoFlo, Abbot Animal Health, Abbot Park, IL, USA) in oxygen. A 3 cm coelomic incision was made at approximately 80 per cent of the distance from the snout to the vent. Surgical exposure permitted visualization of both gonads. The surgical site was flushed with 0.9 per cent sterile saline, and the incision closed.

3. RESULTS AND DISCUSSION

Through DNA fingerprinting, unequivocal genetic differences between alleles present in the offspring and those of the potential *B. c. imperator* sires were observed at multiple loci (table 2), excluding these males as potential sires and ruling out hypothesis 1. All offspring were found to be differentially homozygous at each of the mothers' heterozygous loci, and identical at all loci for which she was homozygous (table 2). This would only be possible through sexual reproduction if the 2004 male possessed the same genotype as the female at each locus screened and contributed the same alleles as the female to each of the offspring. The probability of a zygote receiving identical alleles at a given locus from each heterozygous parent is $p = 0.25$. Across the four maternally heterozygous loci, the probability of an individual receiving identical alleles is $p = 0.0039$ (i.e. $(0.25)^4$). The probability of all individuals within each litter receiving identical alleles across the four loci is: litter $1{-}p = 1.238 \times 10^{-29}$ (i.e. $(0.0039)^{12}$), and litter $2{-}p = 8.14 \times 10^{-25}$ (i.e. $(0.0039)^{10}$). Combined, this gives an overall probability of $p = 1.008 \times 10^{-53}$ (i.e. $(0.0039)^{22}$). Due to this infinitesimally small probability, in the absence of DNA sample from the sire of the 2004 litter, hypothesis 2 (i.e. sexual reproduction) can be rejected. With the exclusion of hypotheses 1 and 2, these results strongly support hypothesis 3 (i.e. facultative parthenogenesis)

Table 1. Characterization of six microsatellite DNA loci developed for *Boa constrictor*. Annealing temperature (T_a), primer concentration, approximate PCR product size (*bp*) and allele number detected are described.

locus	repeat motif	sequence	T_a (°C)	µM each primer	mM $MgCl^2$	fragment size (*bp*)	no. of alleles observed	accession no.
Bci-14	$(AAGA)^{26}$ $(AGGA)^{27}$	F: GCTGTGGTTGGTAAGCAA	60	0.75	1.5	295	5	HM536628
		R: AAGACAGAACCCAGCCAA						
Bci-15	$(TATC)^{20}$	F: GGCTCATCTCAAAGGAA	57	0.9	2.5	256	3	HM536629
		R: CCTTCTCTGCATTGGAA						
Bci-18	$(TCCT)^{21}$	F: GCAAATACCCTCTGAGCA	54	1.5	2.5	297	2	HM536630
		R: AGTTAAGCCATCGGCCTA						
Bci-21	$(AG)^{12}$	F: TTGAGAGATGGACCTGGA	57	0.5	2.5	269	2	HM536631
		R: AGAGACGGAGATACCCAA						
Bci-23	$(TCTG)^5$ $(TC)^3$	F: CAGTACGGGCCATTAGCA	57	0.25	2.5	223	3	HM536632
		R: CTGGGTGAGTTCCATGGA						
USAT20	$(CTC)^6$	F: GTTTCTTCCAGTCTGTATGCCG	54	0.85	2.5	255	4	see Tzika *et al.* [3]
		R: ATGCCAAGGGCCGGA						

Table 2. Genotypes of the mother, four potential *B. c. imperator* sires and 22 female offspring at eight microsatellite loci.

snake	*Bci*-14	*Bci*-15	*Bci*-18	*Bci*-21	*Bci*-23	USAT1	USAT20	USAT36
mother	295/295	256/260	297/301	269/269	223/227	373/373	255/255	310/322
male 1	267/303	260/260	297/297	269/279	223/227	365/365	249/270	306/318
male 2	287/287	260/260	297/301	269/269	227/235	365/373	249/270	314/314
male 3	287/321	260/268	297/297	269/269	235/235	376/376	243/243	310/314
male 4	303/321	260/268	297/297	269/269	235/235	376/376	255/255	310/314
2009-OS 1	295/295	256/256	301/301	269/269	227/227	373/373	255/255	310/310
2009-OS 2	295/295	256/256	297/297	269/269	223/223	373/373	255/255	310/310
2009-OS 3	295/295	260/260	301/301	269/269	223/223	373/373	255/255	310/310
2009-OS 4	295/295	256/256	297/297	269/269	227/227	373/373	255/255	322/322
2009-OS 5	295/295	260/260	301/301	269/269	223/223	373/373	255/255	322/322
2009-OS 6	295/295	256/256	301/301	269/269	223/223	373/373	255/255	310/310
2009-OS 7	295/295	260/260	297/297	269/269	223/223	373/373	255/255	322/322
2009-OS 8	295/295	256/256	301/301	269/269	227/227	373/373	255/255	322/322
2009-OS 9	295/295	260/260	297/297	269/269	227/227	373/373	255/255	322/322
2009-OS 10	295/295	256/256	297/297	269/269	227/227	373/373	255/255	322/322
2009-OS 11	295/295	256/256	297/297	269/269	223/223	373/373	255/255	310/310
2009-OS 12	295/295	256/256	297/297	269/269	227/227	373/373	255/255	322/322
2010-OS 1	295/295	256/256	301/301	269/269	223/223	373/373	255/255	322/322
2010-OS 2	295/295	256/256	301/301	269/269	227/227	373/373	255/255	322/322
2010-OS 3	295/295	256/256	297/297	269/269	223/223	373/373	255/255	322/322
2010-OS 4	295/295	256/256	301/301	269/269	223/223	373/373	255/255	322/322
2010-OS 5	295/295	260/260	297/297	269/269	227/227	373/373	255/255	310/310
2010-OS 6	295/295	260/260	301/301	269/269	223/223	373/373	255/255	310/310
2010-OS 7	295/295	256/256	301/301	269/269	223/223	373/373	255/255	322/322
2010-OS 8	295/295	260/260	297/297	269/269	223/223	373/373	255/255	322/322
2010-OS 9	295/295	256/256	297/297	269/269	227/227	373/373	255/255	322/322
2010-OS 10	295/295	260/260	301/301	269/269	223/223	373/373	255/255	310/310

as the mechanism responsible for the production of these offspring.

Owing to homomorphism of the sex chromosomes within the Boidae, gender cannot be determined from chromosomal morphology [19]. Sex was, therefore, determined as female through cloacal probing. Owing to the monetary and scientific value of these snakes, this was confirmed in a single specimen by exploratory surgery to visualize the gonads. Based upon the presence of two ovaries and the absence of testes, the morphological gender of the parthenogenetically produced snake was determined to be female. At this stage of development in the sexually produced males examined, both testes and ovaries were present, whereas in the sexually produced females only the ovaries were present.

The elevated level of homozygosity observed in the offspring suggests that the parthenogenetic mode may be terminal fusion automixis. Resulting from the fusion of the second polar body to the egg nucleus, homozygosity is observed across all chromosomes, including those involved in sex determination [2]. The absence of males in these litters is puzzling, as terminal fusion should result in equal ratios of ZZ to WW. Maternal hemizygosity for the W sex chromosome (i.e. WO), resulting from chromosomal non-disjuntion at conception, followed by terminal fusion automixis would explain the all-female litters. Without further investigation, however, this is purely speculation. Regardless, such genome-wide homozygosity is of concern from a conservation viewpoint, owing to the fixation of potentially deleterious gene combinations that may lead to a reduction in evolutionary adaptability. As the offspring are non-clonal, limited intra-litter variation will exist, however, owing to maternal heterozygosity at some loci. Previous studies suggest that only ZZ zygotes resulting in male offspring will develop [3,5,6,12,20]; however, their long-term viability is unknown as many fail to hatch or are stillborn (e.g. [5]). The reproductive ability of the offspring documented here is yet to be determined; however, if fertile, all offspring resulting from sexual reproduction will be female (ZW), elevating heterozygosity and permitting the subsequent production of males.

These findings describe the first evidence of multiple, viable, non-experimentally induced WW females in a vertebrate lineage, contradicting reports of inviability previously documented in domestic fowl [4]. Additionally, this is the first scientifically confirmed record of facultative parthenogenesis in a primitive serpent of the family Boidae. Uniquely, in contrast to all other reports of reptilian parthenogenesis in captivity, this female produced offspring only in years when males were present. Although courting activity with males was observed, copulation was not, making it impossible to determine the factors stimulating parthenogenetic reproduction in this species. The non-clonal nature of the offspring rules out oocyte activation following sperm inactivation [2].

The detection of facultative parthenogenesis within this primitive serpent broadens our knowledge of the occurrence of automictic parthenogenesis in vertebrates. Distinctively, it identifies viable WW females, provoking the possibility that WW females may exist in other basal reptilian lineages. The unusual context

in which these were produced suggests that within the primitive constricting snakes, parthenogenesis may be more common than we previously assumed.

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