

Shark Virgin Birth Produces Multiple, Viable Offspring

KEVIN A. FELDHEIM, DEMIAN D. CHAPMAN, DOUG SWEET, SEÁN FITZPATRICK, PAULO A. PRODÖHL, MAHMOOD S. SHIVJI, AND BOB SNOWDEN

- 5 From the Pritzker Laboratory for Molecular Systematics and Evolution, The Field Museum, 1400 South Lake Shore Drive, Chicago, IL 60605 (Feldheim); the Institute for Ocean Conservation Science, School of Marine and Atmospheric Science, Stony Brook University, Stony Brook, NY 11794-5000 (Chapman); the London State Fish Hatchery, Division of Wildlife, Ohio Department of Natural Resources, 2470 Robert's Mill Road SW, London, OH 43140 (Sweet); the School of Biological Sciences, Queen's University Belfast, Belfast, BT9 7BL, Northern Ireland, UK (Fitzpatrick and Prodöhl); The
10 Guy Harvey Research Institute, Oceanographic Center, Nova Southeastern University, Dania Beach, FL 33004 (Shivji); and the Pittsburgh Zoo & PPG Aquarium, One Wild Place, Pittsburgh, PA 15206 (Snowden).

Address correspondence to Kevin A. Feldheim at the address above, e-mail: kfeldheim@fieldmuseum.org.

15 Facultative automictic parthenogenesis has only recently been confirmed in the most ancient jawed vertebrates, the chondrichthyan fishes (sharks, batoids, and chimeras). To date, however, in both documented cases, the females have only produced a single parthenogen offspring, and none of these have lived for more than 3 days. We present a genetically verified case of automictic parthenogenesis by
20 a white-spotted bamboo shark (*Chiloscyllium plagiosum*), in which at least 2 parthenogens were produced and survived for 5 years or more. These findings demonstrate that some female sharks are capable of producing, multiple, viable offspring through parthenogenesis.

25 **Key words:** AFLPs, microsatellites, parthenogenesis, white-spotted bamboo shark

30 There is growing interest in the evolutionary significance and conservation implications of facultative parthenogenesis in vertebrates, as well as the potential biomedical applications that could result from understanding the underlying mechanisms involved (Lampert 2008). Though rare, cases of spontaneous parthenogenesis have now been documented in all major vertebrate lineages other than mammals (e.g., sharks [Chapman et al. 2007], teleost fish [Lampert et al.
35 2007], reptiles [Schuett et al. 1998; Groot et al. 2003; Watts et al. 2006], and birds [Olsen 1975]). Most of these appear to be the result of fusion of postmeiotic products (i.e., the ovum and a polar body: “automictic parthenogenesis”), which produces diploid offspring that are either homozygous for one or the other maternal alleles at many loci (through “terminal fusion”) or that are genetically identical or nearly identical to the mother (through “central fusion,”
40 Lampert 2008). Automictic parthenogenesis was only recently genetically verified in chondrichthyan fish (sharks,

45 batoids, and chimeras; Chapman et al. 2007, 2008). In the first case, a newly mature female bonnethead shark (*Sphyrna tiburo*) that had been wild captured as a young-of-the-year and thereafter isolated from conspecific males in captivity for more than 3 years produced a single, live female offspring. This offspring was homozygous for a maternal allele at 4 polymorphic microsatellite loci (Chapman et al. 2007) and only survived for 3 days before being killed by another fish in the aquarium. In the second case, a well-developed female embryo was discovered during the necropsy of female blacktip shark (*Carcharhinus limbatus*)
50 that had also been wild captured as a juvenile and subsequently isolated from males for 9 years. The embryo was homozygous for a maternal allele at 6 polymorphic microsatellite loci (Chapman et al. 2008). In summary: 1) verified cases of parthenogenesis in chondrichthyan fish are restricted to sharks of the Order Carcharhiniformes that exhibit placental viviparity, 2) in both cases, the offspring exhibited complete homozygosity at the microsatellite loci that were typed, 3) there is no evidence that females can produce multiple offspring from parthenogenesis, and 4) the verified cases provide no information on long-term viability of sharks produced in this manner. These issues make it difficult to assess the biological significance of parthenogenesis in sharks.
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70 In 1996, the Belle Isle Aquarium of the Detroit Zoological Institute acquired one female brown-banded bamboo shark (*Chiloscyllium punctatum*; ISIS accession no. 5458) and one female white-spotted bamboo shark (*Chiloscyllium plagiosum*; ISIS accession no. 5455) as donations from 2 private aquarium hobbyists. Each bamboo shark was purchased by the hobbyist as an egg case and was hatched and reared with no contact with other bamboo sharks. Both animals were sexually immature when first acquired by the
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Table 1. Genotypes of a bamboo shark mother (ISIS accession no. 5455) and 2 of her pups at 10 microsatellite loci

Shark	Cp1 (11 ^a)	Cp3 (6 ^a)	Cp4 (5 ^a)	Cp5 (6 ^a)	Cp7 (4 ^a)	Cp8 (6 ^a)	Cp9 (12 ^a)	Cpl80 (4 ^b)	Cpl150 (4 ^b)	Cpl471 (3 ^b)
Mother	315/315	228/228	377/377	418/422	176/176	283/283	200/208	265/293	202/202	224/226
Pup1	315/315	228/228	377/377	422/422	176/176	283/283	208/208	265/265	202/202	226/226
Pup2	315/315	228/228	377/377	422/422	176/176	283/283	208/208	293/293	202/202	226/226

^a Number of alleles observed in a sample of 33–35 individuals taken from the wild near Zhanjiang, China (Ding et al. 2009).

^b Number of alleles observed in a sample of 9 aquarium-bred individuals in the United States (for loci Cpl80, Cpl150, and Cpl471).

80 Belle Isle Aquarium. Because bamboo sharks are plentiful in zoological park facilities, there was no intent on breeding these individuals, and no males were ever added to cohabitate with these 2 females. In fact, there were no males of either species or any closely related species housed anywhere in the facility. The brown-banded bamboo shark never oviposited eggs. The white-spotted bamboo shark, on the other hand, oviposited egg cases on numerous occasions over a 6-year period. None of these egg cases were saved, as they were presumed to be infertile, until in early 2002 the Curator of Fishes (D.S.) heard about the virgin birth of a bonnethead shark, *S. tiburo*, at the Henry Doorly Zoo in Omaha, Nebraska (later genetically confirmed by Chapman et al. 2007). At this time, D.S. initiated an experiment to demonstrate that this female was reproducing in the absence of males (i.e., by parthenogenesis). Unfertilized eggs cases laid by this female were collected and incubated to see if they would develop into viable embryos. In 2005, genetic testing was initiated to 1) verify an absence of paternal genetic material in the offspring and 2) test the hypothesis that the offspring exhibit elevated homozygosity relative to their mother, as has been observed for other sharks (e.g., Chapman et al. 2007, 2008).

70 Seven eggs were deposited by the *C. plagiosum* between March and August of 2002. All 7 were saved and incubated for 15–16 weeks. Four of them developed embryos that went to full term (15 weeks) before hatching. All embryos were females and were kept in a separate tank from the mother. Three of the four individuals were vigorous within the egg case and hatched without assistance; the fourth appeared to be weakening as it surpassed its expected hatch date, and therefore, its emergence was assisted by excising the shark from the egg case. This last individual (ISIS accession no. 10901) never initiated feeding and only lived for 14 days. The other 3 individuals (ISIS accession nos 10846, 10892, and 10895) were fully viable and grew well during their first year of life. One of these individuals (10846) perished after jumping from its tank at 15 months of age. Unfortunately, the 2 offspring that did not survive were not tissue sampled before being discarded. In contrast, the other 2 remaining offspring continued growing and were subadult size by May of 2005 at that time they were tissue sampled (via small clips of fin). The 2 surviving offspring and the mother were eventually transferred from the closing Belle Isle Aquarium to Denver's Landry's Downtown Aquarium and held in a tank with a large group of conspecific individuals, which precluded further tracking of these sharks.

We used microsatellites and amplified fragment length polymorphism (AFLP) to survey the genome of the suspected parthenogens to identify distinct nonmaternal alleles and potentially refute parthenogenesis. Seven variable microsatellite loci (Cp1, Cp3, Cp4, Cp5, Cp7, Cp8, and Cp9) described elsewhere (4–12 alleles/locus, Ding et al. 2009) and 3 new loci (Cpl80, Cpl150, and Cpl471) developed using an enrichment protocol (Glenn and Schable 2005) were used to genotype the mother and her 2 subadult offspring. To assess variability of the new loci, 9 captive animals (3 from the Pittsburgh Zoo and 6 from the University of South Florida) were also genotyped. All 3 loci amplified alleles in all 9 individuals and exhibited 3 (Cpl471) or 4 (Cpl80 and Cpl150) alleles. Microsatellite genotypes of all individuals, including the mother and her offspring, are shown in Table 1. Multilocus AFLP was also used to characterize the genetic diversity of the offspring. AFLP screening was carried on a LI-COR dual-laser system after the protocol of Zenger et al. (2006) using the selective primers in 14 different combinations (details available on request). Resulting fragments were scored and analyzed (band sharing) using the GeneProfiler software (Scanalytics Inc., Fairfax, VA).

The microsatellite and AFLP genotypes of the *C. plagiosum* offspring are consistent with their suspected parthenogenetic origin and are characteristic of terminal as opposed to central fusion automixis (Lampert 2008). Each proved to be homozygous at every microsatellite locus for one or the other of the possible maternal alleles (Table 1). Like the microsatellite analysis, all AFLP fragments observed in the suspected parthenogen offspring were also present in the mother (i.e., there were no unique paternal fragments in the offspring). Furthermore, both offspring displayed a reduced number of fragments in comparison to the mother (1.36% and 2.31% fewer fragments than the mother), and they differed in 1.74% of their fragments. The combined microsatellite and AFLP data set failed to reveal unique paternal genetic variation. Combining these genetic data with the well-documented captive history of the mother indicating lifetime isolation from males, the evidence that these births were due to parthenogenesis is thus unequivocal. The elevated homozygosity of the 2 offspring relative to the mother reflects previous cases of parthenogenesis in sharks and implies that terminal fusion automixis is the probable parthenogenetic mechanism (Chapman et al. 2007, 2008).

Notably, the 9 aquarium bred *C. plagiosum* we tested, including the mother, appeared to be from inbred lines.

175 Each individual exhibited a high proportion (~50%) of
 homozygous microsatellite loci, and several loci known to
 be polymorphic in a wild population (Ding et al. 2009) were
 monomorphic in the 9 captive animals we tested (e.g.,
 180 mother in Table 1). Multilocus AFLP analysis supports the
 inbred status of the aquarium-bred individuals: they shared
 an average of 91.5% of AFLP fragments, despite being from
 different institutions. This is higher than what has been
 observed for other shark species. For instance, Chapman
 et al. (2007) showed that unrelated wild-captured bonnet-
 185 head sharks (*S. tiburo*) share <69% of their AFLP fragments
 generated using similar methodology. Even given the
 background levels of homozygosity in the captive animals,
 the parthenogens were the only individuals that exhibited
 complete homozygosity at the 10 microsatellite loci, and
 190 there were a sufficient number of polymorphic AFLP
 markers (148) to have shown paternal alleles if they were
 present.

Though genetically untested, it is highly probable that
 the unsampled third and fourth of the hatched offspring
 195 were also parthenogens, which means that up to 4 of 7 eggs
 in this clutch (57%) developed asexually in the absence of
 sperm. This is the first genetically confirmed report of
 multiple offspring being produced by parthenogenesis by
 any female chondrichthyan. Moreover, the discovery of
 200 automictic parthenogenesis in *C. plagiosum* extends the
 occurrence of this form of reproduction from the Order
 Carcharhiniformes to the nonsister Order Orectolobi-
 formes. Thus, automictic parthenogenesis is now known
 to occur in taxonomically diverse shark species with repro-
 205 ductive modes ranging from oviparity to placental viviparity
 (Chapman et al. 2007, 2008). It is therefore reasonable to
 speculate that more shark species are capable of asexual
 reproduction on occasion. Although sperm storage has been
 demonstrated in many species of shark (Pratt 1993), our
 210 findings suggest that inferences of long-term sperm storage
 in captive female sharks producing offspring, despite
 isolation from males (e.g., Castro et al. 1988), should be
 considered suspect unless genetic testing can exclude
 parthenogenesis.

215 Very little is known about the viability of automictic
 parthenogens in sharks (Chapman et al. 2007, 2008).
 Although the elevated homozygosity of automictic parthe-
 nogens could reduce their fitness by increasing expression of
 recessive genetic disorders and decreasing genomic diversity,
 220 long-term viability of automictic parthenogens has been
 reported in teleost fish, reptiles, and birds (Olsen 1975;
 Schuett et al. 1998; Watts et al. 2006; Lampert et al. 2007).
 We document that sharks also can produce viable offspring
 in this manner. Unfortunately, the closure of the Belle Isle
 225 aquarium and the disbursement of the sharks preclude an
 assessment of whether or not these parthenogens are also
 fertile.

Although the present case is the only genetically
 documented instance of parthenogenesis in *C. plagiosum*, we
 230 are aware of other cases of possible parthenogenesis by captive
C. plagiosum females (e.g., Voss et al. 2001, <http://www.azcentral.com/news/articles/1113phx-shark1114.html>). This

species is the only elasmobranch where there is currently more
 than one report of possible parthenogenesis by a female in
 captivity; as such it may prove to be a useful model
 235 elasmobranch to study the mechanism of facultative parthe-
 nogenesis in fish.

The ability of captive *C. plagiosum* to produce multiple
 viable offspring through parthenogenesis may be related
 to the high levels of homozygosity and presumed inbred
 240 lines we observed in these aquarium animals. Hedrick
 (2007) noted that the likelihood of producing viable
 embryos by automictic parthenogenesis is greatly reduced
 when lethal recessive alleles are present in the genome.
 We speculate that inbred individuals, with elevated
 245 homozygosity relative to wild conspecifics, are already
 “purged” of highly deleterious and lethal recessive alleles
 to a certain extent, which could enhance their ability to
 produce viable offspring through automictic partheno-
 genesis. Automictic parthenogenesis might then be ex-
 250 pected to result in viable offspring more often in inbred
 captive populations and, perhaps, in genetically depauper-
 ate wild populations of endangered species than in outbred
 lines.

We show that *C. plagiosum* is capable of automictic
 255 parthenogenesis and provide the first evidence in sharks
 that multiple offspring can be produced in this manner
 and that they can live for long periods (>5 years). We
 suggest that other reported cases of female *C. plagiosum*
 producing viable offspring in the absence of males are also
 260 likely to be the result of parthenogenesis. The demon-
 stration of parthenogenesis in a third lineage of sharks raises the
 prospect that this reproductive capability may not be
 uncommon in these ancient fishes.

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