

Parthenogenesis in a large-bodied requiem shark, the blacktip *Carcharhinus limbatus*

D. D. CHAPMAN*†, B. FIRCHAU‡ AND M. S. SHIVJI§

*Pew Institute for Ocean Science, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Cswy, Miami, FL 33149, U.S.A., ‡Virginia Aquarium & Marine Science Center, 717 General Booth Boulevard, Virginia Beach, VA 23451, U.S.A. and §Guy Harvey Research Institute, Oceanographic Center, Nova Southeastern University, 8000 North Ocean Drive, Dania Beach, FL 33004, U.S.A.

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Genetic evidence is provided for parthenogenesis in a large-bodied shark, the blacktip *Carcharhinus limbatus*, from the speciose and commercially important family Carcharhinidae, the first verified case of asexual development in this lineage and only the second for any chondrichthyan. The parthenogenetic embryo exhibited elevated homozygosity relative to its mother, indicating that automictic parthenogenesis is the most likely mechanism. Although this finding shows that parthenogenesis is more common and widespread in sharks than previously realized and supports the early existence of parthenogenetic abilities in vertebrates, the adaptive significance of automixis in these ancient fishes remains unclear.

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Automictic parthenogenesis (automixis) is a type of asexual reproduction characterized by fusion of an ovum and its sister polar body, producing a diploid zygote with elevated homozygosity compared to its mother (Schuett *et al.*, 1998). Experimental evidence for vertebrate automixis was recently obtained for a bony fish (Lampert *et al.*, 2007), and it is considered very likely to be the mechanism often underlying facultative parthenogenesis in more derived vertebrate lineages [reptiles and birds (Olsen, 1975; Schuett *et al.*, 1998; Watts *et al.*, 2006; Lampert *et al.*, 2007)]. Automixis is also postulated as the mechanism behind the first confirmed case of parthenogenesis in the most ancient jawed vertebrate lineage, the Chondrichthyes (sharks, batoids and chimeras), where a parthenogenetic embryo with elevated homozygosity was recently described in a small-bodied hammerhead shark *Sphyrna tiburo* (L.) (Sphyrnidae) (Chapman *et al.*, 2007). Since automixis is easily overlooked in wild vertebrate populations and there is only a rudimentary understanding of its breadth of evolutionary occurrence and frequency (Chapman *et al.*, 2007;

†Author to whom correspondence should be addressed. Tel.: +1 305 421 4908; fax: +1 305 421 4077; email: dchapman@rsmas.miami.edu

Lampert *et al.*, 2007), it is currently of general biological interest to determine how widespread and common it is among sharks.

There are a growing number of instances where captive female sharks have produced apparently normally developed offspring despite extended periods of isolation from conspecific males (Castro *et al.*, 1988; Voss *et al.*, 2001; Heist, 2004), suggesting that asexual development could be more common and evolutionarily widespread in this lineage than reflected by the single genetically verified case in *S. tiburo* (Chapman *et al.*, 2007). Almost all these suspect cases involve oviparous (egg-laying) species that have small adult body sizes (<1.2 m total length, L_T). Although this may reflect a bias towards the practicality of keeping smaller shark species in captivity, it could also indicate that small-bodied sharks have evolved parthenogenesis as a means to avoid reproductive failure in situations when males are scarce within isolated habitat patches since small shark species tend to have more limited dispersal capabilities than larger species (Musick *et al.*, 2004). An intriguing possible case of parthenogenesis in a large-bodied, highly migratory shark was revealed on 30 May 2007 during the necropsy of *c.* 9 year-old captive female blacktip shark *Carcharhinus limbatus* (Müller & Henle) that had failed to fully revive after being tranquilized during a routine veterinary examination. The necropsy revealed a single, well-developed female embryo, even though the adult female had been isolated from conspecifics for all 8 years of its captivity. Exhibiting placental viviparity, female *C. limbatus* typically reach sexual maturity around age 7 years (Killam & Parsons, 1989) and give birth to multiple offspring every other year after a gestation period of 12 months (Castro, 1996). This indicates that the embryo was probably produced during the first or second ovulation of this female. The adult female had shared the display tank with only one other carcharhiniform shark, an adult male sandbar shark *Carcharhinus plumbeus* (Nardo) that was also captured locally. Despite daily observations by aquarium curators and routine veterinary examinations of the female, there was never any physical evidence of copulation between these two sharks (*i.e.* mating wounds or observations of mating).

Given the captivity circumstances and the case of automixis in the carcharhiniform *S. tiburo* (Chapman *et al.*, 2007), the hypothesis that the *C. limbatus* embryo had resulted from automictic parthenogenesis was tested (*i.e.* with the expectation that it would have no paternal *C. limbatus* or *C. plumbeus* alleles and would exhibit elevated homozygosity rather than being an exact genotypic match to its mother). Tissue samples (fin clips) were obtained from the *C. limbatus* mother and her embryo and stored in 95% ethanol. Following genomic DNA isolation (DNeasy kit; Qiagen Inc., Valencia, CA, U.S.A.), five microsatellites (three to 20 alleles per locus) previously isolated from the genome of *C. limbatus* were amplified in both individuals (locus-specific protocols and diversity are given by: Keeney & Heist, 2003). The polymerase chain reaction (PCR) products were resolved on an AB 3130 DNA analyser and scored in the programme GENEMAPPER 3.7 (Applied-Biosystems Inc., Foster City, CA, U.S.A.). All reactions were replicated and the genotypes scored by two experienced DNA analysts.

The embryo's composite five-locus genotype contained no paternal alleles and every locus exhibited homozygosity for a maternal allele (Table I), both findings

concordant with automixis. The probability of obtaining an embryo that is homozygous for all five loci assuming sexual reproduction within the source *C. limbatus* population (U.S. Atlantic Ocean) was estimated by multiplying the frequencies of observed homozygosity from population genetic data given in Keeney *et al.* (2005). This probability is extremely low ($P < 0.0001$), permitting rejection of an alternate hypothesis of sexual reproduction between a male *C. limbatus* and the mother while she was a small juvenile prior to capture followed by an extraordinarily long period of sperm storage. The alternate hypothesis that the embryo was sired by the male *C. plumbeus* can also be rejected because four of the five microsatellites amplify both of these carcharhinid species (Keeney & Heist, 2003) with the expectation that paternal *C. plumbeus* alleles should be observable in the embryo's composite genotype. Although it would have been desirable to have also genotyped the *C. plumbeus* to confirm that it amplifies at these loci, the animal is too large to remove safely from the tank for DNA sampling. It originated, however, from the same *C. plumbeus* population where these loci were shown to cross amplify (Keeney & Heist, 2003). A species-specific *C. plumbeus* PCR-primer (Pank *et al.*, 2001) failed to amplify genomic DNA from the embryo, while a *C. limbatus*-specific primer succeeded, further verifying that the embryo is not a hybrid of these two species.

The genetic results coupled with the captive history of the mother make automixis the most tenable explanation for the embryo's development. This finding provides the second verified case of parthenogenesis in chondrichthyans and the first for any large-bodied species or from within the commercially important and speciose family Carcharhinidae, thus extending the known evolutionary breadth of asexual reproduction in these ancient fishes. This finding also supports the early existence of parthenogenetic abilities in vertebrates and makes it plausible that the growing number of other reported but genetically unverified cases of reproduction by a diverse range of female chondrichthyans in the extended absence of conspecific males may be the result of automictic parthenogenesis as opposed to sperm storage. Automixis in *C. limbatus* also provides a second instance of parthenogenesis in a placentally viviparous shark species, raising further questions about the relationship between placental reproduction and evolution of genomic imprinting, as has been proposed for mammals (Haig, 2004; Chapman *et al.*, 2007).

Automixis is probably rare in wild populations of *C. limbatus* with robust gender ratios. For example, assuming production of a single embryo is typical

TABLE I. Microsatellite genotypes of the mother *Carcharhinus limbatus* (M) and embryo (E). Individual allele sizes (bp) include a labelled M13 primer. Locus designations are from Keeney & Heist (2003)

Locus	M	E
<i>Cli100</i>	234/234	234/234
<i>Cli13</i>	212/232	232/232
<i>Cli107</i>	127/129	127/127
<i>Cli108</i>	150/152	152/152
<i>Cli7</i>	205/205	205/205

of automictic development in sharks (Chapman *et al.*, 2007; present study), only one out of 221 gravid female *C. limbatus* (0.45%) examined by scientists at the Natal Sharks Board in South Africa between 1978 and 2006 was documented to have been gestating a lone embryo and even this case could have resulted from processes other than parthenogenesis (*e.g.* abortion of other embryos during capture; S. Winter, pers. comm.). What is unclear, however, is whether automictic development of unfertilized ova in sharks is an occasional aberration in the ova or a facultative response of the shark to an absence of suitable mates. The latter appears to be the case in some reptiles in which some captive females have regularly switched between sexual reproduction and asexual reproduction according to the presence or absence of males (Watts *et al.*, 2006). Although this female *C. limbatus* reproduced *via* automixis during what was most likely its first ovulation and small oviparous sharks have produced several offspring in the absence of males on multiple occasions, it remains unknown whether automixis can be a repeated, facultative response to an absence of males in sharks. Regardless of how it occurs, the widespread population collapses occurring for many sharks due to overexploitation (Baum *et al.*, 2003; Baum & Myers, 2004; Robbins *et al.*, 2006; Myers *et al.*, 2007) may increase the expression of automixis if females have difficulty finding mates at low population densities and significant numbers of their ova are left unfertilized.

Whether the automictic development of unfertilized ova is selectively advantageous in sparse or strongly female-biased vertebrate populations is an open question. Although it is intuitively appealing that the ability to reproduce asexually would be selectively advantageous for females in situations where males are sparse, this may not always be the case. Automictic parthenogens have reduced genetic diversity (elevated homozygosity), with potentially reduced fitness consequences (Schuett *et al.*, 1998; Watts *et al.*, 2006; Chapman *et al.*, 2007). The genetic costs of automixis might offset the benefit of having a mechanism to avoid occasional reproductive failure in increasingly sparse, overexploited populations of large-bodied carcharhinids.

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