

**BRIEF COMMUNICATION****Long-term sperm storage in the brownbanded bamboo shark *Chiloscyllium punctatum***M. A. BERNAL\*†‡, N. L. SINAI†§, C. ROCHA†, M. R. GAITHER†||,  
F. DUNKER† AND L. A. ROCHA†

\*University of Texas Marine Science Institute, 750 Channel View Dr, Port Aransas, TX 78373, U.S.A., †California Academy of Sciences, 55 Music Concourse Dr, San Francisco, CA 94118, U.S.A., §School of Veterinary Medicine, University of California, Davis, CA 95616, U.S.A. and ||School of Biological and Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, U.K.

(Received 12 May 2014, Accepted 13 November 2014)

This study investigated the birth of a brownbanded bamboo shark *Chiloscyllium punctatum* at the Steinhart Aquarium. Genetic analyses suggest this is the longest documented case of sperm storage for any species of shark (45 months).

© 2014 The Fisheries Society of the British Isles

Key words: next-generation sequencing; parthenogenesis; RAD-Seq; shark conservation.

On January 2012, a pup of *Chiloscyllium punctatum* Müller & Henle 1838 was born at the Steinhart Aquarium of the California Academy of Sciences (CAS; Fig. 1). What makes this event unusual is that one female of *C. punctatum* laid this egg after at least 45 months of complete isolation from males. The last time the three potential mothers of the pup could have been in contact with a male was at the Aquarium of the Pacific, from which they were acquired in September 2007. The three females were moved to the temporary location of the Steinhart Aquarium in downtown San Francisco, where they started producing eggs immediately. These eggs were not closely monitored at the time and were eventually discarded. A year later, the females were moved to new aquarium facilities in Golden Gate Park, in an exhibit where all other shark specimens are female blacktip reef sharks *Carcharhinus melanopterus* (Quoy & Gaimard 1824), and the only male individual is a Javanese cownose ray *Rhinoptera javanica* Müller & Henle 1841. Recent studies have documented hybridization between closely related chondrichthyans (Morgan *et al.*, 2012) but the phylogenetic distance between the male *R. javanica* and the female *C. punctatum* is such that successful reproduction would be highly improbable. After a few weeks in the exhibit, the females started producing up to six egg cases per week. It was not possible to determine which females produced

‡Author to whom correspondence should be addressed. Tel.: +1 415 379 5277; email: bernal.moises@utexas.edu



FIG. 1. Pup of *Chiloscylium punctatum* born at the Steinhart Aquarium of the California Academy of Sciences. Photograph by Timothy Wong.

the eggs or how many eggs each female produced. Starting on January 2011, egg cases were collected from the enclosure and relocated to a separate shark egg exhibit, where their development was monitored. Two of the egg cases started showing embryonic development on 16 November 2011. One of the eggs hatched on 21 January 2012, while the other did not fully develop. Considering that the females had not been in contact with males for an extended period, there are two hypotheses that could explain the origin of the pup: (1) long-term sperm storage by one of the female *C. punctatum* or (2) facultative parthenogenesis, which has been previously demonstrated for the closely related whitespotted bamboo shark *Chiloscylium plagiosum* (Bennett 1830) (Feldheim *et al.*, 2010).

Several species of sharks are known to store sperm from months to years (Pratt, 1993). Long-term sperm storage is an advantageous strategy, as fertilization can occur even if females are not ovulating at the time of insemination (Holt & Lloyd, 2010). While the mechanism of storage has not been studied in *C. punctatum*, it is known from other shark species that sperm is stored in the tubules of the terminal zone of the oviducal gland (Pratt, 1993; Storrie *et al.*, 2008). These glands are expanded regions of the anterior portion of the oviduct and are important in the production of jelly that surrounds fertilized eggs (Hamlett *et al.*, 2002). If the pup was a product of fertilization between an egg and stored sperm, it should have unique alleles inherited from the father, which are not present in any of the potential mothers.

The alternative explanation for births in the absence of males is parthenogenesis. In vertebrates, facultative parthenogenesis occurs in sexually reproducing species when females spend extended periods of time in the absence of males. Four species of sharks are known to reproduce *via* facultative parthenogenesis: *C. plagiosum* (Feldheim *et al.*,

2010), bonnethead sharks *Sphyrna tiburo* (L. 1758) (Chapman *et al.*, 2007), blacktip sharks *Carcharhinus limbatus* (Müller & Henle 1839) (Chapman *et al.*, 2008) and zebra sharks *Stegostoma fasciatum* (Hermann 1783) (Robinson *et al.*, 2011). In these cases, females had been isolated from males for years and the origin of the offspring was validated by genotyping. The cases documented thus far correspond to automictic parthenogenesis, in which a diploid zygote is formed by the fusion of an ovum and a polar body. This results in a zygote with low allelic diversity and elevated rates of homozygosity, compared with one produced via sexual reproduction (Chapman *et al.*, 2007, 2008; Feldheim *et al.*, 2010).

To test these contrasting hypotheses, the three potential mothers were genotyped, as well as the pup, using polymorphic loci obtained *via* restriction site-associated DNA sequencing (RAD-Seq). A small piece of fin was obtained from each individual of *C. punctatum* by the aquarium staff during their annual physical examination. DNA was extracted from each tissue following a standard phenol–chloroform extraction protocol. After extraction, RAD-Seq loci were obtained following the double digest protocol of Peterson *et al.* (2012). The prepared libraries were sequenced at the Vincent J. Coats Genomics Sequencing Laboratory of UC Berkeley on an Illumina HiSeq 2000 (www.illumina.com; 100 bp single end reads). After de-multiplexing, reads with Phred scores <33 were discarded. Fragments containing single nucleotide polymorphisms (SNP) were identified using the STACKS pipeline (Catchen *et al.*, 2013). With the Population programme implemented in STACKS, the dataset was filtered to obtain loci that were only present in all individuals with coverage of 10× or higher. The dataset was exported in Structure file format (Pritchard *et al.*, 2000). Using GenoDive, clonal distance was calculated between the four individuals, which estimates the number of mutational steps that are needed to transform the genotype of one individual into the genotype of another individual (Meirmans & Van Tienderen, 2004). The number of heterozygous sites for each individual was determined with Microsoft Excel, using the formula =if (A1=A2, 1, 0), which gives a value of 1 to homozygous and 0 to heterozygous sites. The totals for each individual were calculated and the homozygous sites were subtracted from the total number of loci to obtain the number of heterozygous sites. Heterozygosity was estimated by dividing the number of heterozygous sites by the total number of loci.

After quality control and filtering, 154 loci were retained. The clonal distance analysis demonstrated that the potential mothers had between 59 and 74 mutational steps of separation (Table I). The pup was the most dissimilar individual of the four, being 73 steps different from female A, 98 steps from female B and 94 steps from female C (Table I). The similarity of the pup with female A suggests this is the mother of the

TABLE I. Clonal distance between individuals of *Chiloscyllium punctatum* from the Steinhart Aquarium

	Female A	Female B	Female C	Pup
Female A	–	67	59	73
Female B	67	–	74	98
Female C	59	74	–	94
Pup	73	98	94	–

TABLE II. Number of heterozygous alleles and heterozygosity for individuals of *Chiloscyllium punctatum* from the Steinhart Aquarium

Individual	Heterozygous alleles	Heterozygosity
Female A	62	0.40
Female B	73	0.47
Female C	63	0.41
Pup	69	0.44

pup. Of the 154 loci, the pup was heterozygous at 69 of them (44%), while the three adult females were heterozygous at 62, 73 and 63 sites (Table II). Thus, the pup had levels of heterozygosity comparable with the three females: a finding that is incongruent with expectations of automictic parthenogenesis, where individuals are expected to have lower heterozygosity than their mother (Chapman *et al.*, 2007; Feldheim *et al.*, 2010). Most importantly, 32 private alleles were found in the pup that were found in none of the adult females (Table III). The best explanation for the presence of these alleles is that they were inherited from the father of the pup, most likely a male from the Aquarium of the Pacific. Unfortunately, there is no information on the whereabouts of the sharks that shared the enclosure with the three females from this study.

The evidence suggests that this is a case of long-term sperm storage in *C. punctatum*. Since the eggs were deposited in the summer of 2011, one female of *C. punctatum* (most likely female A) stored sperm for a period of at least 45 months. This scenario is plausible if the biology of *C. punctatum* and the history of the individuals of the Steinhart Aquarium are considered. First, observations of *C. punctatum* in captivity have revealed that there is a single period of mating per year between July and September, and females lay eggs from July to February of the following year (Harahush *et al.*, 2007). Thus, individuals of *C. punctatum* are known to regularly store sperm for at least 7 months. Moreover, the total length of the three females upon arrival to the Steinhart Aquarium was 58.4, 67.3 and 66.0 cm, and one of them was gravid at the time of transfer from Aquarium of the Pacific to the Steinhart Aquarium (these eggs did not develop). Females of *C. punctatum* are reported to be sexually mature at 62 cm (Compagno, 2001), which means at least two of the females had been sexually mature before they arrived at the Steinhart Aquarium. Even if not fully mature, studies of the

TABLE III. Example of 10 private alleles found on the pup of *Chiloscyllium punctatum*

	428_1	556_12	1471_76	1489_60	2885_69	3511_73	4344_51	5811_33	5855_45	5872_51
Female A	2	3	3	4	4	1	3	3	3	3
	2	3	3	4	4	1	3	3	3	3
Female B	2	3	3	4	4	1	3	3	3	3
	2	3	3	4	4	1	3	3	3	3
Female C	2	3	3	4	4	1	3	3	3	3
	2	3	3	4	4	1	3	3	3	3
Pup	2	1	1	1	1	1	1	3	1	3
	3	3	3	4	4	2	3	4	3	4

reproductive tract of female gummy sharks *Mustelus antarcticus* Günther 1870 suggest that maturing females are also able to store sperm (Storrie *et al.*, 2008).

Previous studies on sharks have reported sperm storage periods of 13 (Storrie *et al.*, 2008) to 28 months (Castro *et al.*, 1988). Considering females had not been in contact with males for at least 45 months, this is the longest documented case of sperm storage and successful fertilization for any species of shark. Previous cases of parthenogenesis in sharks brought to light an interesting challenge for their conservation: as shark populations decline, finding mates could become more difficult, which could lead to an increase of facultative parthenogenesis. Even if parthenogenesis becomes more common in natural populations due to overfishing and habitat loss, long-term sperm storage may also play an important role in maintaining genetic diversity of dwindling shark populations.

The authors would like to acknowledge T. Wong who provided Fig. 1 and the staff of the Center of Comparative Genomics and the Steinhart Aquarium, especially B. Simison, A. Sellas and J. Arase, for their support with this project. This study was possible with the financial support of the California Academy of Sciences to L.A.R. and SENACYT to M.A.B.

## References

- Castro, J. I., Bubucis, P. M. & Overstrom, N. A. (1988). The reproductive biology of the chain dogfish, *Scyliorhinus retifer*. *Copeia* **1988**, 740–746.
- Catchen, J., Hohenlohe, P., Bassham, S., Amores, A. & Cresko, W. (2013). Stacks: an analysis tool set for population genomics. *Molecular Ecology* **22**, 3124–3140.
- Chapman, D. D., Shivji, M. S., Louis, E., Sommer, J., Fletcher, H. & Prodöhl, P. A. (2007). Virgin birth in a hammerhead shark. *Biology Letters* **3**, 425–427.
- Chapman, D. D., Firchau, B. & Shivji, M. S. (2008). Parthenogenesis in a large-bodied requiem shark, the blacktip *Carcharhinus limbatus*. *Journal of Fish Biology* **73**, 1473–1477.
- Compagno, L. J. V. (2001). Sharks of the world: an annotated and illustrated catalogue of shark species known to date. *FAO Species Catalogue for Fishery Purposes* **1**.
- Feldheim, K. A., Sweet, D., Fitzpatrick, S., Prodöhl, P. A., Shivji, M. S. & Snowden, B. (2010). Shark Virgin birth produces multiple, viable offspring. *Journal of Heredity* **101**, 374–377.
- Hamlett, W. C., Fishelson, L., Baranes, A., Hysell, C. K. & Sever, D. M. (2002). Ultrastructural analysis of sperm storage and morphology of the oviducal gland in the Oman shark, *Iago omanensis* (Triakidae). *Marine and Freshwater Research* **53**, 601–613.
- Harahush, B. K., Fischer, A. B. P. & Collin, S. P. (2007). Captive breeding and embryonic development of *Chiloscyllium punctatum* Muller & Henle, 1838 (Elasmobranchii: Hemiscyllidae). *Journal of Fish Biology* **71**, 1007–1022.
- Holt, W. V. & Lloyd, R. E. (2010). Sperm storage in the vertebrate female reproductive tract: how does it work so well? *Theriogenology* **73**, 713–722.
- Meirmans, P. G. & Van Tienderen, P. H. (2004). GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* **4**, 792–794.
- Morgan, J. A. T., Harry, A. V., Welch, D. J., Street, R., White, J., Geraghty, P. T., Macbeth, W. G., Tobin, A., Simpfendorfer, C. A. & Ovenden, J. R. (2012). Detection of interspecies hybridisation in Chondrichthyes: hybrids and hybrid offspring between Australian (*Carcharhinus tilstoni*) and common (*C. limbatus*) blacktip shark found in an Australian fishery. *Conservation Genetics* **13**, 455–463.
- Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S. & Hoekstra, H. E. (2012). Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS One* **7**, 37135. doi: 10.1371/journal.pone.0037135
- Pratt, H. L. (1993). The storage of spermatozoa in the oviducal glands of western North Atlantic sharks. *Environmental Biology of Fishes* **38**, 139–149.

- Pritchard, J. K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **15**, 945–959.
- Robinson, D. P., Baverstock, W., Al-Jahru, A., Hyland, K. & Khazanehdari, K. A. (2011). Annually recurring parthenogenesis in a zebra shark *Stegostoma fasciatum*. *Journal of Fish Biology* **79**, 1376–1382.
- Storrie, M. T., Walker, T. I., Laurenson, L. J. & Hamlett, W. C. (2008). Microscopic organization of the sperm storage tubules in the oviducal gland of the female gummy shark (*Mustelus antarcticus*), with observations on sperm distribution and storage. *Journal of Morphology* **269**, 1308–1324.