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BRIEF COMMUNICATION

Parthenogenesis in a whitetip reef shark *Triaenodon obesus* involves a reduction in ploidy

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Genetic analysis of a female whitetip reef shark *Triaenodon obesus* and her stillborn pup, assumed to be of parthenogenetic origin, revealed that the pup was homozygous at all 24 nuclear-encoded microsatellites assayed, consistent with the idea that diploidy in the pup had been restored *via* terminal fusion. Flow cytometric analysis, however, indicated that the genome size of the pup was no more than half that of the mother, and microscopy revealed that nuclear volume was *c.* 1·73 times larger in the mother than in the pup. Together these data suggest that the pup was genetically haploid, developing directly from an unfertilized egg; as far as is known, this is the first observation of a spontaneously produced haploid vertebrate.

Key words: elasmobranchs; haploid; virgin birth.

Facultative parthenogenesis, the production of offspring by females in the absence of males, has been documented in *c.*70 species of vertebrates (Watts *et al.*, 2006). There have been a number of theories put forward to explain the evolutionary benefit of switching from sexual to asexual reproduction (Lampert, 2008). In apomictic parthenogenesis, meiosis is repressed and offspring are full clones of their mother (Pearcy *et al.*, 2011). This mode of reproduction has some fitness advantages for females capable of engaging in facultative parthenogenesis that allows them to reproduce when sex ratios are not favourable, while at the same time passing on their full complement of genes (Neaves & Baumann, 2011). The majority of vertebrates documented to engage in facultative parthenogenesis, however, appear to use automixis (diploidization after completion of meiosis) as the mode for restoring diploidy (Lampert, 2008). Several automictic processes (*e.g.* gametic duplication, central and terminal fusion) can restore

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diploidy but differ in the extent that heterozygosity is reduced in the progeny relative to the mother (Pearcy *et al.*, 2011). Because females engaging in automictic parthenogenesis pass on as little as half of their genetic material, there is an expected increase in homozygosity and expression of recessive lethals (Hedrick, 2007); consequently, benefits to this reproductive mode are unclear. Finally, eggs may develop directly into haploid offspring; this strategy appears to be common in invertebrates (Normark, 2003) but is not well known in vertebrates outside a laboratory setting (Hamilton, 1963).

Parthenogenesis has been confirmed, using genetic data, in four species of captive elasmobranchs, all in situations where a female had been isolated from male conspecifics for >3 years. In two cases, one involving a bonnethead shark *Sphyrna tiburo* (L. 1758) and the other a blacktip shark *Carcharhinus limbatus* (Müller & Henle 1839), a single pup was found to be completely homozygous for three microsatellites that were heterozygous in the mother (Chapman *et al.*, 2007, 2008). In the third case, two offspring from a single female whitespotted bamboo shark *Chiloscyllium plagiosum* (Anonymous [Bennett] 1830) were completely homozygous at four microsatellites heterozygous in the mother (Feldheim *et al.*, 2010). In the fourth case, involving a zebra shark *Stegostoma fasciatum* (Herman 1783), three offspring were genotyped (two embryos and one neonate). One embryo and the neonate were completely homozygous at nine microsatellites that were heterozygous in the mother. The other embryo was heterozygous at one of the microsatellites heterozygous in the mother; however one of the two alleles present in the pup at that microsatellite was not present in the mother (Robinson *et al.*, 2011). These studies provide evidence that parthenogenesis in sharks involves a reduction in heterozygosity, consistent with automixis but also consistent with what would be expected from a genetically haploid individual. A more thorough evaluation of the biological processes behind parthenogenesis thus requires an evaluation of heterozygosity loss and a verification of ploidy level. This would provide insight as to whether a parthenogenetic reproductive strategy in sharks can increase individual female fitness and implications as to the occurrence of parthenogenesis in wild populations of sharks.

In June 2012, a female whitetip reef shark *Triaenodon obesus* (Rüppell 1837) gave birth to a single, developed, pre-term female pup at the BioPark Aquarium, in Albuquerque, New Mexico. The pup was found dead by aquarists and the cause of death was undetermined. There were no obvious signs of trauma, suggesting the animal had been stillborn. Subsequent examination of the specimen did not reveal any obvious morphological abnormalities. The pup was immediately frozen until tissues were sampled. The specimen, tissue samples, as well as tissue samples from the mother, are currently housed in the Museum of Southwestern Biology at University of New Mexico (MSB 86005). The mature female was acquired from the Monterey Bay Aquarium in 2006 as a sub-adult (*c.* 1·2 m), and since that time had no contact with conspecific males. From 2006 to 2009, the mother was housed in the same tank with a spotted wobbegong shark *Orectolobus maculatus* (Bonnaterre 1788), and an ornate wobbegong shark *Orectolobus ornatus* (De Vis 1883); both sharks were female. The female *T. obesus* was then isolated from all other sharks between 2009 and the birth of the pup.

Tissue samples of mother and pup, preserved in 10% dimethyl sulphoxide buffer (Seutin *et al.*, 1991), were obtained from the museum. DNA was extracted following a modified Chelex extraction protocol (Estoup *et al.*, 1996). After a final 2 min centrifugation at 13 000 *g*, 1–2 µl of supernatant was used as a template for PCRs. A total of 24 microsatellites, originally isolated from the confamilial blacknose

TABLE I. Genotypes of a female *Triaenodon obesus* and her stillborn pup at 11 microsatellites that were heterozygous in the mother

	cac1	*cac34*	*cac42*	*cac57*	*cac68*	*cis157*	*cis18*	*cis4*	*cis107*	*cis175*	*mca31*
Female	210/212	212/214	268/278	200/208	161/163	225/267	163/165	245/247	275/287	171/201	232/308
Pup	212/212	214/214	278/278	200/200	163/163	225/225	163/163	247/247	287/287	201/201	232/232

shark *Carcharhinus acronotus* (Poey 1860) and finetooth shark *Carcharhinus isodon* (Müller & Henle 1839), as well as from the triakid, dusky smooth-hound *Mustelus canis* (Mitchill 1815), were amplified in both mother and pup. The forward primer from each primer pair was labelled with one fluorescent label of Dye Set D (Applied Biosystems; www.appliedbiosystems.com): 6-FAM, HEX or NED. Descriptions of primers and protocols of PCR amplifications may be found in studies by Giresi *et al.* (2012*a*, *b*, *c*). Amplicons were electrophoresed on 6% polyacrylamide gels, using an ABI Prism 377 sequencer (Applied Biosystems) and GeneScan™ 400HD ROX™ Size Standards (Applied Biosystems) in each lane. Scoring was conducted manually, using GeneScan 3.1.2 (Applied Biosystems) and GenoTyper 2.5 (Perkin Elmer; www.perkinelmer.co.uk).

Of the 24 microsatellites screened, the mother was heterozygous for 11; the pup was homozygous for a maternal allele at all 24 microsatellites (Table I). This is a 100% reduction in heterozygosity for all markers assayed. There are several automictic mechanisms that can restore diploidy: central fusion with recombination, terminal fusion with recombination, terminal fusion without recombination and gametic duplication; the last two generate extreme heterozygosity loss (Suomalainen *et al.*, 1987; Lampert *et al.*, 2007). The microsatellite data, however, also are consistent with a reduction from diploidy to haploidy, something not considered in previous investigations.

Genome sizes (DNA content) of both mother and pup were estimated using methods described by Hare & Johnston (2011). Briefly, nuclei were liberated by macerating *c.* 1 mm^2 of fin tissue in Galbraith buffer, using sterile razor blades. Fresh fruit fly *Drosophila virilis* (heads), prepared the same way, was used as a DNA standard. *Triaenodon obesus* and *D. virilis* were further ground with 20 strokes of a pestle in a 2 ml Dounce homogenizer (Kontes; www.kimble-chase.com). Samples were then filtered through 40 µ nylon mesh and stained with 25 $\mu g\,ml^{-1}$ of propidium iodide. Since propidium iodide intercalates with the major groove of DNA, cells with more DNA content (large genome size) will take up more stain and produce greater levels of fluorescence than cells with less DNA content (small genome size). Samples and standard were, therefore, scored for the amount of fluorescence, using a Partec CyFlo equipped with a green laser (www.partec.com). Tissues of mother and pup were run in replicate. The amount of DNA in the mother was calculated by taking the ratio of fluorescence for the major peak of each replicate (scored average channel numbers 201·9 and 227·7, respectively) and the *D. virilis* standard (set to channel number 25) and multiplying by the DNA content in *D. virilis* (656 Mb). The averaged, estimated diploid genome size of the mother was 5631 Mb (*c.* 2·88 pg C), consistent with estimates of genome sizes in other carcharhinids (Gregory, 2013). DNA content profiles of the pup, on the other hand, were fairly flat for both replicates, a condition typically seen in tissues where DNA had degraded. The highest number of consistent counts for the pup in both replicates (scored average channel numbers 110 and 102·5,

TABLE II. Results of analyses to confirm ploidy of a female *Triaenodon obesus* and her still born pup

Microscopy*	Size	S.E.
Light		
Mother	5·10	0·50
Pup	4·20	0·24
Confocal		
Mother	5·11	0·55
Pup	4·32	0·39
Cytometry†	**Size**	**Mean**
$Mother_1$	5517·5	
$Mother_2$	5744·0	5630·8
Pup_1	2886·4	
Pup_2	2689·6	2788·0

*Sizes for light and confocal microscopy are presented in microns.
†Genome sizes based on flow cytometry are presented in Mb of DNA.

respectively), however, indicated that the pup contained no more than half the genome size (2788 Mb estimated) of the diploid mother.

As a secondary assessment of ploidy level, the diameter of 20 interphase nuclei were measured from cells of both mother and pup, using an ocular micrometer and phase-contrast optics. Mean ± S.E. diameter of nuclei from the mother measured $5{\cdot}1 \pm 0{\cdot}24$ μm while those from the pup measured $4{\cdot}2 \pm 0{\cdot}5$ μm. Nuclear size also was assessed in both mother and pup, using confocal microscopy. Mean ± S.E. cross-sectional size for nuclei from the mother was $5{\cdot}11 \pm 0{\cdot}55$ μm, while that from the pup was $4{\cdot}32 \pm 0{\cdot}39$ μm. These measures produce approximate nuclear volumes of 1218 μm^3 for the mother and 705 μm^3 for the pup, resulting in a 1·73:1 ratio of maternal to pup nuclear volume. Given that nuclear volume is expected to increase by *c.* x1·59 with a doubling in DNA content (Cavalier-Smith, 1978), these measurements along with results of flow cytometry suggest that the pup is genetically haploid. A summary of cytometric and microscopic results can be found in Table II.

The microsatellite results are consistent with previous findings in elasmobranchs that documented a complete reduction in heterozygosity, albeit with fewer heterozygous markers documented in the mother (Chapman *et al.*, 2007, 2008; Feldheim *et al.*, 2010). The single exception was a *S. fasciatum* embryo that was heterozygous at one microsatellite, although one of the alleles at that microsatellite was not present in the mother (Robinson *et al.*, 2011). Unlike prior studies, however, the results suggest that the observed homozygosity in this case is not the result of automixis (terminal fusion) but rather the result of a reduction in ploidy level. It is important to note that the original goal of the project was simply to verify parthenogenesis and document reduction in heterozygosity; consequently, tissues were not preserved in an ideal way for cytometry or microscopy. Nonetheless, all measures of ploidy and estimates of nuclear volume were consistent with a two-fold reduction in genome size in the pup relative to the mother. This, as far as is known, is the first report of a fully

developed, haploid vertebrate that occurred spontaneously [Humphrey & Fankhauser (1957), describe spontaneous haploids that were created during experimental crosses or through temperature based manipulation; in the present study nothing experimental was undertaken].

Whether previously reported cases of parthenogenesis in sharks involved a reduction in ploidy level remains unknown. It is important to note that a completely homozygous half clone and a haploid have the same potential reduction in viability and the level to which viability is compromised has implications for maternal fitness. In this case, the *T. obesus* pup appears to have been stillborn; in other instances, genetically homozygous sharks have been live born, succumbing to mortality later (Chapman *et al.*, 2007). In *C. plagiosum*, several documented homozygous offspring lived for several years, although the fate of these animals is unknown as they were distributed to other facilities (Feldheim *et al.*, 2010). Perhaps the most promising data on viability of such animals comes from *S. fasciatum* where a single female produced 30 embryos over a 4 year period in the absence of males (Robinson *et al.*, 2011). The mortality rate across those litters was very high 87% (26/30), but four of the pups apparently were alive when Robinson *et al.* (2011) was published.

The significance of facultative parthenogenesis for wild populations of sharks remains a topic of debate. Facultative parthenogenesis has been observed in two snakes in the genus *Agkistrodon* (Booth *et al.*, 2012), but has only been observed in sharks held in captivity for long periods of time ($\geq$3 years) and in the absence of conspecific males. Data from litters used for paternity analysis in wild populations of sandbar shark *Carcharhinus plumbeus* (Nardo 1827) and spiny dogfish *Squalus acanthias* L. 1758 (Portnoy *et al.*, 2007; Verissimo *et al.*, 2011) were examined and no evidence was found of completely homozygous pups or pups with only maternal alleles. It is also important to note that while certain reptiles produce viable males *via* parthenogenesis (Watts *et al.*, 2006; Booth *et al.,* 2012), all parthenogenetically generated sharks reported to date have been female. Consequently, in a male-limited setting, production of female, homozygous half-clones or haploids would only further skew the sex ratio. Further, while facultative asexual reproduction in theory might accelerate loss of recessive lethals in inbred captive populations (Hedrick, 2007), haploid progeny produced by predominately diploid species also would be expected to express recessive lethals and thus have greatly reduced fitness in wild, outbred populations. While females' production of haploid female progeny may be a mechanism for passing on genes when no other options are available, the persistence of this strategy would necessitate that at least some portion of haploid offspring produce viable gametes. This will need further investigation. In summary, the occurrence and viability of facultative parthenogenesis in wild populations of sharks require further evaluation.

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