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First evidence of multiple paternity in the bull shark (*Carcharhinus leucas*)

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Abstract. The present study assessed the occurrence of multiple paternity in four litters of bull shark *Carcharhinus leucas* ($n = 5, 8, 9$ and 11 embryos) sampled at Reunion Island in the Western Indian Ocean. Using 21 microsatellite loci, we revealed that two litters were generated from two sires each, demonstrating for the first time multiple paternity for this species. We also reported a high paternal skew ($10 : 1$ in Litter 1 and $7 : 1$ in Litter 3), which may be because of post-copulatory or post-zygotic selection processes. These results contribute to a better understanding of the reproductive behaviour of the bull shark, which remains poorly documented. The present study must be expanded to assess the frequency of multiple paternity in this species, and to test for genetic or cryptic benefits (convenience polyandry), which is important for long-term conservation and management plans.

Introduction

Compared with teleosts, elasmobranchs are usually characterised by low fecundity, low growth rates and late sexual maturity (Myers *et al.* 1999; Frisk *et al.* 2001; Dulvy *et al.* 2008) and are therefore very sensitive to overexploitation (Musick *et al.* 2000; Ferretti *et al.* 2010; Dulvy *et al.* 2014). Their populations are also slow to rebound from depletion (Smith *et al.* 1998; Myers and Worm 2005). Consequently, knowledge of their reproductive behaviour is important for developing management and conservation plans (Neff and Pitcher 2002; Rowe and Hutchings 2003). A wide variety of reproductive systems in elasmobranchs has been described, ranging from viviparity to oviparity (Compagno 1990, 2001; Conrath *et al.* 2012), and their mating systems are similarly diverse, with monoandrous and polyandrous species, as well as parthenogenesis being recorded occasionally for captive individuals (Chapman *et al.* 2007, 2008; Portnoy *et al.* 2014). Overall, mating systems in sharks remain poorly documented, because they are difficult to study. Although direct observations suggested multiple matings in the nurse shark (*Ginglymostoma cirratum*) (Pratt and Carrier 2001) and in the whitetip reef shark (*Triaenodon obesus*) (Whitney *et al.* 2004), only genetic tools can confirm multiple paternity, identifying full and half-siblings in the same litter. To date, genetic studies have shown multiple paternity in several elasmobranch species, including the small-spotted cat shark (*Scyliorhinus canicula*) (Griffiths *et al.* 2012), lemon shark

(*Negaprion brevirostris*), sandbar shark (*Carcharhinus plumbeus*), bonnet head shark (*Sphyrna tiburo*) and brown smooth hound (*Mustelus henlei*) (for a review, see Byrne and Avise 2012). Although multiple paternity is common in sharks, variability is observed at both inter- and intraspecific scales (Chapman *et al.* 2004; Feldheim *et al.* 2004; Daly-Engel *et al.* 2007; Portnoy *et al.* 2007; DiBattista *et al.* 2008b; Chabot and Haggin 2014) and several hypotheses have been proposed to explain this process, such as indirect benefits or convenience polyandry (Zeh and Zeh 2001; Griffiths *et al.* 2012).

The bull shark (*Carcharhinus leucas*) is a large shark species (up to 3.4 m long) that is widespread in tropical and subtropical coastal waters. Its diet is very diverse, including high trophic-level prey, which suggests that bull sharks are apex predators playing an important role in structuring marine communities (Daly *et al.* 2013) through top-down processes (Myers *et al.* 2007; Heithaus *et al.* 2008). Typical of most elasmobranchs, bull sharks have a long lifespan, around 39 years (Wintner *et al.* 2002), and delayed sexual maturity, estimated between 14 and 18 years (Branstetter and Stiles 1987). Thus, bull sharks are sensitive to both recreational and commercial fishing pressures to which they are subjected and, as a result, are classified as Near Threatened in the International Union of Conservation for Nature's Red List (Simpfendorfer and Burgess 2009; Worm *et al.* 2013). This species exhibits placental viviparity, and each embryo is initially dependent on a yolk sac, which elongates and

attaches to the uterine wall of the mother after a few weeks, forming a yolk sac placenta. For most of gestation, the mother provides nutrients to the embryo via this yolk sac placenta (Jenson 1976; Compagno 1984; Parsons *et al.* 2008). Litter sizes range from one to 13 embryos (Compagno 1984), with most litters between six and eight (Pattillo *et al.* 1997). Recently Nevill *et al.* (2014) reported a litter size of 14 late stage embryos in the Seychelles, which represents the largest litter size recorded to date for *C. leucas*. No sperm storage in the oviductal gland has been identified in this species yet, but it has been demonstrated in other carcharhinid species (Pratt and Carrier 2001; Parsons *et al.* 2008; Fitzpatrick *et al.* 2012). The bull shark is euryhaline and is able to inhabit both marine and freshwater habitats, including lakes, rivers and estuaries (Compagno 1984). The ability of bull sharks to penetrate freshwater seems mostly related to reproduction, when pregnant females migrate to estuarine areas to give birth (Castro 2011). Despite their ability to travel long distances (Kohler and Turner 2001; Simpfendorfer *et al.* 2005; Daly *et al.* 2013; Heupel *et al.* 2015), several studies have highlighted site fidelity in bull shark populations of Florida (Hueter *et al.* 2005; Karl *et al.* 2011), Australia (Tillett *et al.* 2012) and Fiji (Brunnschweiler and Baensch 2011), and even female reproductive philopatry (Karl *et al.* 2011). Nevertheless, to our knowledge, polyandry has never been demonstrated for the species.

Herein, using 20 microsatellite loci newly developed for this species (Pirog *et al.* 2015) and one microsatellite locus developed for the tiger shark *Galeocerdo cuvier* that cross-amplifies in the bull shark (A. Pirog, unpubl. data), we document the occurrence of multiple paternity in four litters of bull sharks from Reunion Island. Possible implications of this polyandry for population dynamics and management of the species are discussed.

Materials and methods

Samples were collected from four gravid bull shark females, as well as from unborn pups contained in whole uteri. These specimens were caught by local fishermen between June 2013 and June 2015 on the west coast of Reunion Island (21°6'S, 55°36'E). Samples consisted of a piece of muscle biopsied on the mother and on each pup ($n = 11, 5, 8$ and 9 pups per brood) and preserved in 95% ethanol. Total length (TL) was measured for each individual and the sex of each pup was recorded.

Total genomic DNA was extracted from small pieces of tissues using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Genotyping was performed using the 20 microsatellite loci recently developed for *C. leucas* (Pirog *et al.* 2015). Moreover, we tested microsatellite loci developed for other shark species and that cross-amplified in the bull shark, namely five developed for *Carcharhinus limbatus* (Cli-007, Cli-106, Cli-107, Cli-108 and Cli-112; Keeney and Heist 2003) and one developed for *G. cuvier* (Gc01; A. Pirog, unpubl. data). Polymerase chain reactions (PCR) were performed following the conditions published by Keeney and Heist (2003) for the five loci isolated from *C. limbatus* (Cli) and Pirog *et al.* (2015) for the others. Amplicons were run on an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA, USA) and allele size was determined using Genemapper v 4.0 (Applied Biosystems, Foster City, CA, USA).

The five loci isolated from *C. limbatus* were not used in further analyses because of important stuttering bands and potential scoring errors. The other microsatellite loci amplified were polymorphic for a population of bull shark from Reunion Island ($n = 41$). In all, 21 microsatellite loci were used in subsequent analyses.

The probability of detecting multiple paternity in a litter depends on the polymorphism of the microsatellite loci used (allele frequencies), the number of loci, the number of offspring and putative fathers. This probability was calculated using PrDM (Neff and Pitcher 2002) with six different scenarios. These scenarios were defined according to the number of pups observed in the present study ($n = 5-11$) and the number of fathers recorded in other shark species ($n = 2-4$) with and without paternal skew (number of embryos within a litter sired by each male). Multiple paternity was considered when more than two non-maternal alleles were found in the brood for at least two microsatellite loci. Moreover, full and half-siblings in the litter and putative fathers were inferred using Colony ver. 2.0.4.5 software (Jones and Wang 2010) and a maximum likelihood approach. A polygamous mating system was assumed for both sexes to allow the assignment of half-siblings. A long-run with medium likelihood precision and a genotyping error rate of 1% was performed.

Results

In the four litters studied, the number of embryos ranged from 5 to 11 and the sex ratio was biased towards female predominance (M : F = 5 : 6; 1 : 4; 2 : 6 and 3 : 6). In each litter, all pups were approximately the same size (Table 1), but the mean size of pups from each litter differed significantly (all $P < 0.05$, pairwise Wilcoxon test with Bonferroni correction), except between Litters 1 and 4 ($P = 0.17$, pairwise Wilcoxon test with Bonferroni correction). This may be due to the different gestation stages at which each female was caught and may reflect seasonality in the reproduction of the bull shark. Indeed, it is of note that the mother of Litter 2, with the smallest embryos (as well as the smallest number of embryos), was caught in April, the mothers of Litters 1 and 4 were both caught at the beginning of June, and the mother of Litter 3, with the biggest embryos, was caught at the end of August.

The probability of detecting multiple paternity was high (ranging from 0.75 to 1 according to the number of sires) when broods were comprised of more than eight embryos (Table 2). When broods were comprised of five embryos sired by two males, this probability was lower, especially with paternal skews (0.55 to 0.72; Table 2). Consequently it is possible that for litters with few embryos the microsatellite loci used in the present study may have led to underestimation of the number of sires.

For two litters, more than two paternal alleles were observed for at least two microsatellite loci (Table 3), with three to four paternal alleles recorded. Therefore, at least two fathers were identified for Litters 1 and 3. For Litters 2 and 4, only one sire was identified, because the criterion of more than two paternal alleles was not recovered for any of the loci. Nevertheless, for Litter 2, it is possible that the number of fathers was underestimated because of the low number of embryos and the

Table 1. Characteristics of the four litters of bull shark (*Carcharhinus leucas*) studied from Reunion Island

The mean total length (TL) \pm s.e. is given for each litter (mother not included). Data for mothers are shown in bold

	Date of capture	Sex	TL (cm)
Litter 1			
CIRUN100	06/06/2013	F	285
CIRUN101	06/06/2013	F	48
CIRUN102	06/06/2013	M	47
CIRUN103	06/06/2013	M	48
CIRUN104	06/06/2013	F	45
CIRUN105	06/06/2013	M	45
CIRUN106	06/06/2013	M	50
CIRUN107	06/06/2013	F	46
CIRUN108	06/06/2013	F	46
CIRUN109	06/06/2013	F	46
CIRUN110	06/06/2013	M	48
CIRUN111	06/06/2013	F	46
Mean \pm s.e.			46.8 \pm 0.5
Litter 2			
CIRUN200	28/04/2014	F	301
CIRUN201	28/04/2014	M	35
CIRUN202	28/04/2014	F	36
CIRUN203	28/04/2014	F	32
CIRUN204	28/04/2014	F	32
CIRUN205	28/04/2014	F	32
Mean \pm s.e.			33.4 \pm 0.9
Litter 3			
CIRUN300	26/08/2014	F	295
CIRUN301	26/08/2014	M	66
CIRUN302	26/08/2014	F	66
CIRUN303	26/08/2014	F	64
CIRUN304	26/08/2014	F	67
CIRUN305	26/08/2014	F	67
CIRUN306	26/08/2014	M	68
CIRUN307	26/08/2014	F	67
CIRUN308	26/08/2014	F	68
Mean \pm s.e.			66.6 \pm 0.5
Litter 4			
CIRUN400	02/06/2015	F	272
CIRUN401	02/06/2015	M	45
CIRUN402	02/06/2015	F	46
CIRUN403	02/06/2015	F	46
CIRUN404	02/06/2015	F	46
CIRUN405	02/06/2015	M	44
CIRUN406	02/06/2015	F	47
CIRUN407	02/06/2015	F	47
CIRUN408	02/06/2015	F	47
CIRUN409	02/06/2015	M	46
Mean \pm s.e.			46.0 \pm 0.3

characteristics of the microsatellite loci. For Litter 4, one embryo (CIRUN409) did not express the maternal allele for the locus CII6, because both the mother and embryo were homozygous, expressing alleles 109 and 107 respectively. Because genotyping and extraction of DNA were performed independently several times (twice for extraction and three for genotyping), it seems reasonable to reject the hypothesis of genotyping errors or sample confusion. Moreover, embryos

Table 2. Probability of detecting multiple paternity for the 21 microsatellite loci used under six scenarios varying in number of sires and paternal skews

	Number of embryos			
	5 (Litter 2)	8 (Litter 3)	9 (Litter 4)	11 (Litter 1)
Paternal skews				
Two males (50 : 50)	0.79	0.94	0.96	0.97
Two males (66.7 : 33.3)	0.72	0.9	0.92	0.95
Two males (80 : 20)	0.55	0.75	0.79	0.85
Three males (33.3 : 33.3 : 33.4)	0.91	0.99	0.99	1
Three males (57 : 28.5 : 14.5)	0.84	0.96	0.97	0.99
Four males (25 : 25 : 25 : 25)	0.95	1	1	1

Table 3. Allelic evidence of multiple paternity for two litters of bull shark (*Carcharhinus leucas*) at three microsatellite loci (CI08, CI13 and CI16) for which more than two paternal alleles were detected

Allele sizes are in base pairs. Paternal alleles shown in bold. Asterisks indicate one of two alleles from the sire

	CI08	CI13	CI16
Litter 1			
CIRUN100		112, 116	107, 111
CIRUN101		112, 112	103 , 111
CIRUN102		112* , 116*	107* , 111*
CIRUN103		112, 122	107* , 111*
CIRUN104		116, 122	107* , 111*
CIRUN105		112, 122	107, 107
CIRUN106		112* , 116*	103 , 111
CIRUN107		116, 120	107, 109
CIRUN108		112* , 116*	107* , 111*
CIRUN109		116, 122	107* , 111*
CIRUN110		112* , 116*	103 , 107
CIRUN111		112, 122	103 , 111
Paternal alleles		112, 116? , 120, 122	103, 107, 109, 111?
Litter 3			
CIRUN300	148, 148	112, 112	
CIRUN301	148, 154	112, 116	
CIRUN302	148, 152	112, 122	
CIRUN303	148, 154	112, 122	
CIRUN304	148, 152	112, 116	
CIRUN305	148, 152	112, 116	
CIRUN306	148, 148	112, 112	
CIRUN307	148, 154	112, 122	
CIRUN308	148, 154	112, 122	
Paternal alleles	148, 152, 154	112, 116, 122	

were dissected separately from any other individual, avoiding DNA contamination. Consequently, this singularity seems real.

Finally, for each litter, full and half-siblings were inferred. For Litter 4, to perform the analysis, CIRUN409 was considered to have missing data at the CI16 locus. In the two litters with multiple paternity, only one pup (CIRUN107 for Litter 1 and CIRUN306 for Litter 3) was a half-sib with all other pups

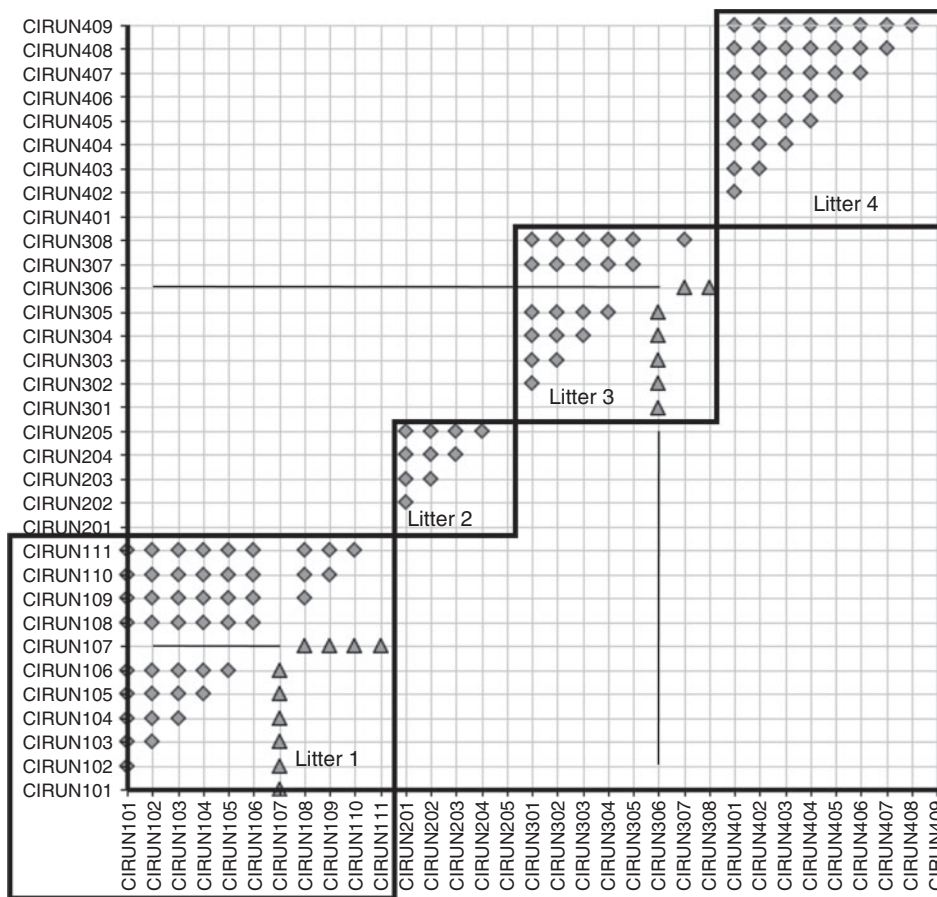


Fig. 1. Sibling relationships (sibships) in the four litters studied. Squares indicate group pups from the same litter. Diamonds (upper diagonal) indicate full siblings; triangles (lower diagonal) indicate half-siblings.

(Fig. 1), confirming that Litters 1 and 3 were fertilised by exactly two males with a high paternal skew (10 : 1 in Litter 1 and 7 : 1 in Litter 3).

Discussion

Herein, we provide the first evidence of multiple paternity in *C. leucas*, with two of four litters being fertilised by two males. Identifying multiple paternity in species is of paramount importance for conservation because it may help maintain genetic diversity in populations (Avise *et al.* 2002; Frankham *et al.* 2010) and may increase estimates of effective population size (Sugg and Chesser 1994; Martinez *et al.* 2000).

Multiple paternity may lead to genetic benefits (for a review, see Thonhauser *et al.* 2014), including fertility assurance, the good gene hypothesis or the genetic diversity hypothesis (Zeh and Zeh 2001; Thonhauser *et al.* 2014). An offspring showing higher genetic diversity would express a better fitness and heterozygosity (DiBattista *et al.* 2008a) and thus these genetic benefits may be detected if multiple paternity is correlated with litter size, heterozygosity or body mass (Zeh and Zeh 2001). Polyandrous litters may be larger than monoandrous litters, as demonstrated for the hammerhead shark (*S. tiburo*) (Chapman *et al.* 2004); however, for several shark species, such as the

lemon shark (*N. brevirostris*) (DiBattista *et al.* 2008a), the brown smooth hound shark (*M. henlei*) (Byrne and Avise 2012) and the leopard shark *Triakis semifasciata* (Nosal *et al.* 2013), no genetic benefits have been demonstrated. In the present study, the sample size was too small to test the correlation between multiple paternity and characteristics of the litters, but it is interesting to note that multiple paternity was not detected in two of four litters, one containing five embryos and the other containing nine embryos. This may suggest that polyandry in the bull shark would not lead to genetic benefits in terms of offspring production, because a smaller litter (eight embryos) was found polyandrous. Nevertheless, a larger sample is required to confirm this observation. Overall, the size of the litters in the present study was in accordance with the average litter size observed in other localities (Compagno 1984; Pattillo *et al.* 1997).

Post-copulatory mechanisms, such as cryptic female sperm choice, are important and may lead to paternal skews in litters (Jennions and Petrie 2000; Fitzpatrick *et al.* 2012; Marino *et al.* 2015). These skews may also be due to post-zygotic processes, as found in the mouse (*Mus musculus*) (Zeh and Zeh 1997), in which females are able to relocate nutrients between defective and viable embryos. Competition between siblings may also occur, such as intrauterine cannibalism described in the fire

salamander (*Salamandra salamandra*) (Dopazo and Alberch 1994) or in the sand tiger shark (*Carcharias taurus*) (Chapman *et al.* 2013). In the present study, an important paternal skew was found in the two broods exhibiting multiple paternity (10 : 1 in Litter 1 and 7 : 1 in Litter 3), which may infer post-copulatory or post-zygotic processes. On the basis of the data of the present study, because the bigger litters were also the most advanced in terms of gestation, it seems unlikely that intrauterine cannibalism occurs, but resource relocation between embryos at a very early stage of gestation may occur. Furthermore, intrauterine cannibalism in sharks has only been reported for *C. taurus* (Chapman *et al.* 2013), a species exhibiting size variation within a brood, whereas in the present study the pups within a litter were of a similar size.

Multiple paternity does not always involve genetic benefits and may be the result of convenience polyandry (Wolff and Macdonald 2004; Holman and Kokko 2013), as demonstrated, for example, in the water strider (*Gerris buenoi*) (Rowe 1992) or in the rock shrimp (*Rhynchocinetes typus*) (Thiel and Hinojosa 2003). Mating of sharks can be violent for females, which show marks and wounds during the mating season (Brunnschweiler and Baensch 2011) resulting from the males grasping females during copulation (Parsons *et al.* 2008). In addition, females also exhibit cloacal lesions because of penetration of the male's clasper (Pratt and Carrier 2001). Avoiding mating could imply a cost for the female, which could be higher than accepting the mating. Under these conditions, females will mate with several males, leading to convenience polyandry (DiBattista *et al.* 2008a; Griffiths *et al.* 2012). This may be the case for the bull shark, because the data of the present study do not infer genetic benefits. If this species aggregates seasonally at specific places to reproduce, then convenience polyandry could be favoured to minimise harassment because the probability of mating should be higher. In this case, polyandry would not lead to any genetic benefit, but would limit the decrease in female fitness resulting from wounds inflicted by males during mating (Holman and Kokko 2013).

Interestingly, one embryo (CIRUN409) did not express the maternal allele for the locus CI16, a dinucleotide (TC) microsatellite (Pirog *et al.* 2015). The mother was homozygous, expressing the allele 109 for this locus, and embryos of the litter were either heterozygous with alleles 107 and 109, or homozygous 109, except for CIRUN409, which was homozygous with the allele 107 (see Table S1, available as Supplementary material to this paper). We can infer from the other embryos of the litter that the father expressed alleles 107 and 109 for this locus. This anomaly may be due to a mutation that occurred in the mother's germ cells (Ellegren 2004; Ortego *et al.* 2008), the presence of a null allele (Dakin and Avise 2004; Chapuis and Estoup 2007) or to meiotic errors (e.g. loss of a chromosome; Engel 1980).

In conclusion, the present study provides new insights into the reproductive behaviour of *C. leucas*, which remains poorly documented. The sampling was insufficient to assess the frequency of multiple paternity in the population of bull sharks of Reunion Island and to test the presence of genetic benefits, but the results tend to support the hypothesis of convenience polyandry. It will be important to expand on this study using non-destructive methods in order to better assess the effects

of polyandry on the genetic diversity and estimates of the effective population size of bull sharks in Reunion Island. Finally, in the context of both shark-attack management and conservation of ecological processes associated with bull sharks, efforts need to continue to understand the full reproductive cycle of the species locally and investigate whether reproduction leads to behavioural changes that could partially explain attacks that happen during the mating period, which is, to date, poorly documented in the western Indian Ocean.

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