Zoo Biology 22:179–187 (2003)

Assessing Reproductive Potential and Gestation in Nurse Sharks (*Ginglymostoma cirratum*) Using Ultrasonography and Endoscopy: An Example of Bridging the Gap Between Field Research and Captive Studies

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Over a period of 3 years, five reproductively active female nurse sharks (*Ginglymostoma cirratum*) from a wild, actively mating population of nurse sharks were captured, confined, and periodically examined through the course of gestation to determine the gestation period and characterize paternity. In the final year of the study, candidate animals were first evaluated in the field by ultrasonography, and the selected animals were then transported from the study site to holding facilities at SeaWorld Adventure Parks in Orlando, Florida. Periodic monitoring of the animals was conducted by ultrasonography, endoscopy, and routine blood analysis. Gestation was determined to be a minimum of 131 days, multiple paternity was shown for two individual litters, and ultrasonography and endoscopy were shown to be useful adjuncts for assessing pregnancy and monitoring gestation in this species. Poor survival of offspring, and small litter size may be a consequence of handling and transporting

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Grant sponsor: Hewlett-Mellon Faculty Development Funds, Albion College; Grant sponsor: W.W. Diehl Endowed Professorship, Albion College; Grant sponsor: Highly Migratory Species Management Division, NOAA.

Received for publication October 17, 2001; Accepted March 6, 2002.

DOI: 10.1002/zoo.10088
Published online in Wiley InterScience (www.interscience.wiley.com).

the animals, and the use of invasive procedures such as endoscopy. Zoo Biol 22:179–187, 2003.

Key words: mating; behavior; reproduction; elasmobranchs

INTRODUCTION

Although several species of large sharks have successfully mated and given birth in captivity, few studies beyond that of Klimley [1980] have systematically detailed the behavioral interactions between mating animals in captivity, and few have followed the reproductive physiology of captive animals. Similarly, comprehensive field studies of the reproductive behavior and biology of large sharks are rare [Pratt and Carrier, 2001]. However, the most complete study to date suggested that behaviors noted in captive animals often differ significantly from those of wild populations [Carrier et al., 1994].

While it may be desirable to conduct studies of natural behaviors such as courtship and mating outside of the captive environment, such studies cannot be controlled and monitored (as is possible in captivity), and they are usually limited in scope because the animals cannot be observed through time. Field observations of individual animals generally do not extend beyond the momentary events of courtship and copulation, and therefore cannot provide even the most basic information, such as the time of gestation. The physiological changes that accompany pregnancy, and that can be detected by routine blood chemistries, are not discernible in field studies. Finally, because nursery grounds are incompletely understood and poorly described for most species of sharks, the assignment of neonates to specific maternal or paternal parents for pedigree analysis is complicated, and would require observers to be present at the birth of a shark to make immediate captures of the mother and littermates.

For the past 11 years, the reproductive behavior of a population of nurse sharks (*Ginglymostoma cirratum*) in the westernmost islands of the Florida Keys has been systematically studied [Carrier et al., 1994; Pratt and Carrier, 2001]. These sharks mate during June at a very specific location that has been known to scientists since the beginning of the last century [Gudger, 1912]. Nurse sharks in the area were tagged by two of the authors (J.C. and H.L.P.) as a part of this ongoing study, and 189 individuals are readily recognizable by unique tags and/or scar patterns. Males have been observed to return to the mating grounds annually, and the study has shown that the females have a 2-year reproductive cycle of mating and parturition. Castro [2000] supported this finding by independent studies utilizing dissection of gravid females taken throughout the year. Studies of nurse shark migration indicate that this species does not show extensive movements [Carrier, 1985; Carrier and Luer, 1990; Kohler et al., 1998]. The presence of neonates and juveniles in all seasons indicates that the area is a nursery ground as well as a mating ground for this species.

Although this study revealed the mechanics of mating and copulation, and the complex behaviors associated with mating [Pratt and Carrier, 2001] in nurse sharks, we were unable to directly measure gestation period or evaluate paternity in this population because of the difficulties inherent in field studies, as noted above. Like most sharks, nurse sharks are long-lived and mobile, and consequently are not restricted to areas where they can be constantly observed.

To address these issues, a collaborative project was initiated with SeaWorld Adventure Parks, Orlando, Florida, to capture actively mating females at the study site and transport selected animals to holding facilities in Orlando, where they were held and monitored throughout gestation. They were later returned with their surviving offspring to the study area at the completion of the captivity portion of the project. Since ultrasonography was used previously as a diagnostic and investigative tool in large elasmobranchs by one of the authors [Walsh et al., 1993] (though not for studies of reproduction), ultrasounds were performed at the field site in the final year of the study to improve the probability that the selected animals were fertile.

METHODS

Female sharks that had been observed in mating activities prior to capture were selected for capture in mid June. Five animals were transported and held in captivity over the course of 3 years (two were taken in year 1 of the study, one in year 2, and two in year 3). Since mating in this species often occurs in waters <2 m deep, the animals were captured with a heavy mesh beach seine (2 m $\times$ 30 m) by surrounding a mating pair and restraining them within the net when the mating event terminated. The males were measured and tagged, blood and tissue samples were obtained, and the animals were immediately released. The females were transferred to a nylon/vinyl sling and either placed in a temporary enclosure at the study site or transferred directly to a chartered research vessel, where they were measured, tagged, and put into a live-animal transport unit (1 m $\times$ 1 m $\times$ 3 m) with oxygen-supplemented, constant-flow circulation.

Sonography was performed in the field in the final year of the study using a Pie medical scanner (Maastricht, The Netherlands) (model 200) with an ASP-18 3.5 MHz linear probe in an attempt to identify egg cases in utero. Animals were selected when the sonography revealed the greatest number of egg cases present in the paired uteri, and they had been observed to mate numerous times throughout the observation period.

Female nurse sharks that were selected for further study were transported by boat to Key West, Florida, where they were then transferred to specially equipped trucks and taken to the SeaWorld facilities at Orlando. Upon arrival, they were immediately transferred to non-display, quarantine pools (either indoor circular pools (12 m diameter $\times$ 1.5 m deep) or in-ground outdoor pools (10 m long $\times$ 4.5 m wide $\times$ 1.5 m deep)). The salinity was maintained at 30–32 ppt, and the temperature was kept at 25°C. Before evidence of parturition was found, the females were separated and sequestered in separate pools. In the final stages of gestation, beginning in mid October, a false bottom constructed of polyvinylchloride (PVC) frames with square openings measuring approximately 10 cm were added to the pools. This permitted spent egg cases to fall to the bottom through the openings, and allowed the neonates to seek refuge and avoid being preyed upon by the females.

The female sharks were fed mackerel and other fish twice weekly in an amount equivalent to 3–5% body weight. To monitor the progress of gestation, animals were removed monthly by sling to a transport unit (1 m $\times$ 1 m $\times$ 3 m) and placed in a supine position under light anesthesia (MS222 at 50 ppm). No further restraint was required.

Blood analyses and ultrasonography were conducted monthly. Examination by endoscopy was performed at 2-month intervals during the same time and under the same anesthesia regime used for sonography. Endoscopy was accomplished using a Corometrics model CMH-150 illuminator and Storz Xenon 300 light source with a Hopkins telescope (5 mm $\times$ 29 cm, 0°) coupled to a Storz veterinary video camera (Hi8). The endoscope was inserted into the cloaca and advanced slowly through the common vagina, anteriorly past the left or right uterine sphincter muscle, to visualize, illuminate, and ultimately photograph the intrauterine environment. Ultrasonography was performed using the same instrument as previously described for the field assessment.

Nurse shark development is characterized as aplacental viviparity (ovoviviparous), and young are known to hatch from eggs held internally in the paired uteri [Castro, 2000]. Empty or nonviable egg cases are released precociously, and young presumably are born 2–3 weeks following the shedding of the case (Perry Gilbert, personal communication). Hence, once egg cases were observed to be present in the holding pools, visual inspections of the tanks by underwater divers and observers on the surface were conducted regularly, several times each day. Egg cases, aborted young, or neonates were then removed from the tank and held in smaller, isolated aquaria where conditions could be more closely controlled and monitored. All observations of shed egg cases, aborted embryos, and births were recorded to help estimate gestation. Neonates were offered fresh clams and shrimp to the point of satiation.

RESULTS

The first two females in year 1 produced no egg cases or young. The third animal (year 2) produced a few egg cases in October and November, but no young were observed. Figure 1 depicts a sonography image of eggs in utero. Both of the

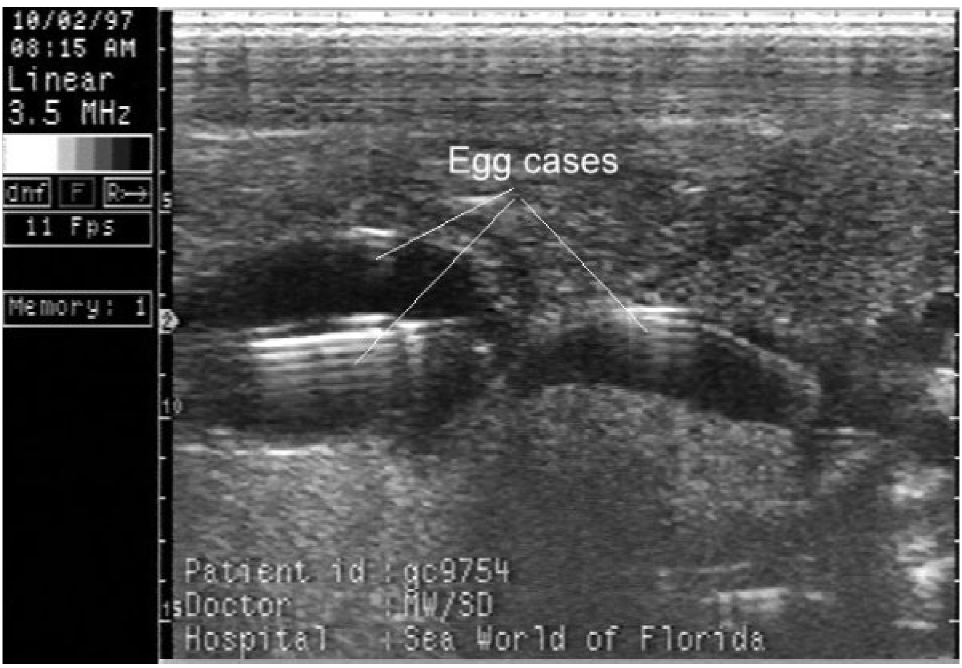

Fig. 1. Ultrasound images of egg cases.

TABLE 1. Year 3 births and egg case recoveries for animals 4 and 5

Female 1D	Offspring	Date of birth	Days lived	Sex	Total length (cm)	Weight (g)	Gestation estimate[a]
9750	Unhatched	9 Oct	0	M	N/A	N/A	131
9750	9750-1	11Oct	0	F	21.4	131	133
9750	9750-2	11 Oct	0	N/A	N/A	N/A	133
9750	9750-3	21 Oct	0	N/A	20	96.2	143
9750	9750-4	16 Dec	0	F	22	N/A	199
9750	9750-5	21 Dec	3	N/A	23.3	81.9	204
9754	9754-1	9 Oct	2	F	21.5	70	131
9754	9754-2	9 Oct	2	F	22.7	118.5	131
9754	9754-3	10 Oct	3	F	21.7	113.4	132
9754	9754-4	11 Oct	1	F	23.6	120.6	133
9754	9754-5	11 Oct	2	F	21.9	89.8	133
9754	9754-6	11 Oct	2	F	22.1	108	133
9754	9754-7	24 Oct	1	N/A	23	87.4	146
9754	9754-8	30 Oct	Released in June	M	24	N/A	152
9754	9754-9	13 Nov	0	M	24	N/A	166
9754	9754-10	19 Nov	9	N/A	25	92.3	172

[a]Estimate of gestation presumes mating begins when maternal or (adult) females first appear in study site showing evidence of mating scars, and is set at June 1.
N/A data not recorded.

animals selected in year 3 produced egg cases, aborted young with egg sacs still attached, and gave birth to young that lived for short durations, as summarized in Table 1. However, only one neonate (9754-8) survived to release. All five adult females were returned to the study site and released during the summer following their capture.

The intrauterine endoscopy of both year 3 animals revealed the presence of egg cases and debris from disintegration of unfertilized eggs. The final endoscopy was performed on October 2, at which time an emergent embryo was visible (Fig. 2) in animal 9750. Simultaneous ultrasounds were performed to verify position and orientation, although the thick skin of female nurse sharks limits image quality (Fig. 3). The movements of the embryo as recorded on the videotape of the procedure indicate that it was alive at the time of the procedure.

The length of gestation ranged from 131 to 204 days. A closer examination of Table 1 shows that the average total length (TL) of this litter was 21.7 cm. The largest animal (9750-5, 23.3 cm) was the last one to be born. Littermates from animal 9754 averaged 22.9 cm TL and had better rates of survival.

DISCUSSION

The recovery of egg cases in year 2 enabled us to make a preliminary estimate of the time of gestation, to allow time to prepare the false bottom and establish observation protocols for animals in year 3. The successful recovery of egg cases, aborted embryos, and neonates in year 3 suggests that the protocol was successful.

Castro [2000] reported litter sizes ranging between 21 and 50 embryos in this species, and an average total length at birth of 28–30.5 cm. The small sizes at birth

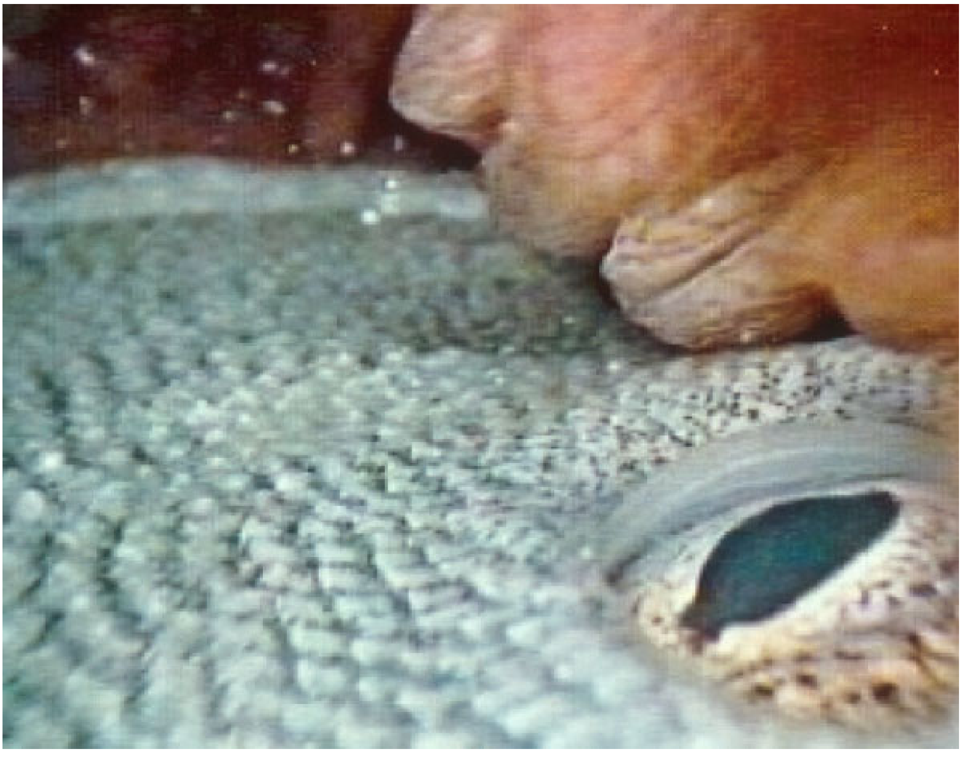

Fig. 2. Intrauterine image from endoscopy showing embryo post-hatching, estimated to be 124 days post-mating. Uterine folds are evident in the upper right corner. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

and poor survival rates in the present study do not agree well with Castro's results, although the natural mortality of neonates is unknown. The differences observed in this study could be attributable to stresses related to capture and handling, to the use of endoscopy at a particularly critical time in gestation, to excessive handling, or to infectious agents introduced during the endoscopic procedures. Despite the appearance of a hatchling during the endoscopic procedure, it could be that the large number of births and aborted embryos that followed within 1–2 weeks may have been caused by this invasive procedure interrupting the normal course of development. Further, it is unlikely that the single survivor (9750-5) was the embryo observed in utero, since it was born 80 days following the procedure, and, as noted previously (Perry Gilbert, personal communication), birth is generally thought to occur within 10–14 days following intrauterine hatching. The imaging produced from an endoscope in *G. cirratum* is far superior to that obtained by ultrasound, as its level of resolution is comparatively superb. However, the use of endoscopy may increase embryo mortality, and should be evaluated prior to use as an assessment procedure. Species with thinner skin might benefit from the use of ultrasound alone, without endoscopy. This is especially important when monitoring the progress of gestation to isolate gravid females, or when other issues related to captive breeding or animal husbandry are critical, and it may minimize the risk of inducing spontaneous abortions or premature births.

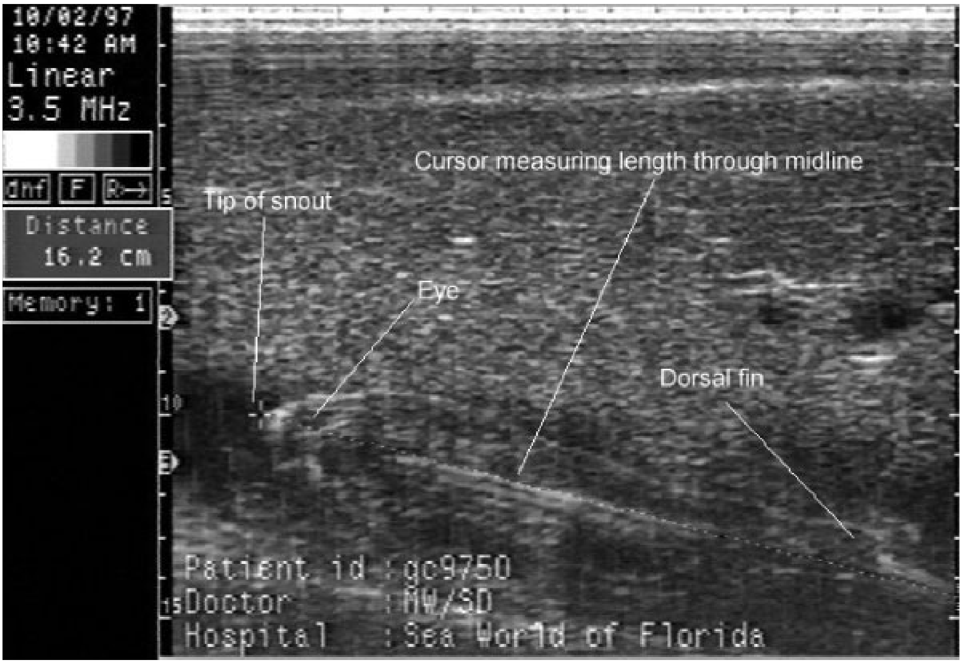

Fig. 3. Sonograph of embryo, post-hatching, estimated to be 124 days post-mating.

The small litter sizes (n=6 and 10) in this study, compared to the median of 34 reported by Castro [2000], likely resulted from early removal of active females from the field. Only those ova in transit from the ovary to the oviducal gland would have been candidates for fertilization when the sharks were captured, and removing animals from the presence of males prevented further introduction of sperm (despite continued production of ova), and a normal litter size under these conditions would therefore be unlikely. Furthermore, it is probable that nurse shark ova are produced serially during estrus, and ovulation may continue for several weeks. Individual large, yolky ova may therefore be fertilized on different occasions, depending upon what sperm might be present at the time, as they descend through the oviducal gland. Since ovulation continues over a period of many weeks, this may explain the range of births observed from early October through late December, and implies that the period of mating may last somewhat longer than the authors reported previously. Later births would presumably result from matings that occurred later in the mating season. This conclusion is consistent with the study by Castro [2000], in which embryos were shown in various developmental stages. Early embryonic death may also explain small litter sizes; however, that could not be confirmed in the current study.

Tissue samples taken from the offspring and mothers, and subsequent DNA analyses indicated multiple paternity in each litter [Saville et al., 2002]. This was as expected, from field observations of females mating with multiple male partners, and is consistent with ovulation that continues for several weeks (since opportunities to mate with multiple males may be more likely). This might also be interpreted as indirect evidence of the absence of sperm storage [Pratt, 1993] or, alternatively, low sperm viability in this species. There could thus be a need for multiple matings in order for successive groups of ova to be fertilized, and paternity may simply be determined by whatever sperm is present when the ova are candidates for fertilization.

CONCLUSIONS

1. The gestation period was determined to be at least 131 days, and may range to as long as 207 days.

2. Field sonography, when combined with visual observations of mating, improves the probability of obtaining animals that are in the early stages of pregnancy.

3. Intrauterine endoscopy is a superior technique for visualizing the progress of gestation, but may carry risks, including spontaneous abortion and accelerated gestation, which could be exacerbated by excessive or rough handling, or introduced infectious agents.

4. Juxtaposing field studies of shark reproduction with studies of reproduction in captivity avoids the problems associated with artificial conditions, and at the same time maximizes the monitoring capabilities that are only possible in captivity. Nevertheless, issues related to transport and maintenance, as well as differences between captive and natural conditions, may produce results that are not fully consistent with observations in the wild.

ACKNOWLEDGMENTS

The authors express their appreciation to SeaWorld Adventure Parks, Orlando, for providing logistical support for the field portion of this study, and for providing facilities and staff for maintenance of the animals in captivity. We particularly thank Dr. S. Dover for his assistance with endoscopy, and J. Kerivan, R. Davis, and all of the aquarists who participated in the capture and maintenance of the nurse sharks. We also thank C. Perry for her clinical expertise, and management of the blood chemistries performed in the field and clinical settings throughout the study. In addition, we are grateful to P. Taylor and the staff of the Dry Tortugas National Park for their suggestions and support, and the staff at the National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Narragansett Laboratory for their help with shark tags and equipment, and support. Finally, we thank C. Carrier and T. Pratt for their very able assistance and continued support throughout the study. Funding was provided to J.C.C. in part by Hewlett-Mellon Faculty Development Funds from Albion College, and the W.W. Diehl Endowed Professorship of Albion College, and to H.L.P. from the Highly Migratory Species Management Division of the NOAA.

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